

Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation

Yuchang Fu,^{1,*} Nanlan Luo,^{*} Richard L. Klein,[†] and W. Timothy Garvey^{*,§}

Department of Nutrition Sciences,^{*} University of Alabama at Birmingham, Birmingham, AL 35294-3360; Department of Medicine,[†] Medical University of South Carolina, and Charleston VA Medical Center, Charleston, SC 29425; and Birmingham VA Medical Center,[§] Birmingham, AL 35233

Abstract Adiponectin is secreted from adipocytes, and low circulating levels have been epidemiologically associated with obesity, insulin resistance, type 2 diabetes, and cardiovascular disease. To investigate whether adiponectin could exert autocrine effects in adipocytes, we expressed the adiponectin gene in 3T3-L1 fibroblasts. We observed that 3T3-L1 fibroblasts expressing adiponectin have a fast growth phase and reach confluence more rapidly compared with control cells or LacZ-transduced cells. Furthermore, cells with overexpressed adiponectin were observed to differentiate into adipocytes more rapidly, and during adipogenesis, they exhibited more prolonged and robust gene expression for related transcriptional factors, CCAAT/enhancer binding protein α (C/EBP α), peroxisome proliferator-activated receptor γ (PPAR γ), and adipocyte determination and differentiation factor 1/sterol-regulatory element binding protein 1c (ADD1/SREBP1c) and earlier suppression of PPAR γ coactivator-1 α (PGC-1 α). In fully differentiated adipocytes, adiponectin-overexpressing cells accumulated more and larger lipid droplets compared with control cells. Also, adiponectin increased insulin's ability to maximally stimulate glucose uptake by 78% through increased glucose transporter 4 (GLUT4) gene expression and increased GLUT4 recruitment to the plasma membrane. These data suggest a new role for adiponectin as an autocrine factor in adipose tissues: promoting cell proliferation and differentiation from preadipocytes into adipocytes, augmenting programmed gene expression responsible for adipogenesis, and increasing lipid content and insulin responsiveness of the glucose transport system in adipocytes.—Fu, Y., N. Luo, R. L. Klein, and W. T. Garvey. Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. *J. Lipid Res.* 2005. 46: 1369–1379.

Supplementary key words obesity/diabetes • gene expression • adipocytokine • glucose • lipid metabolism

Adipose tissue is a highly active metabolic and endocrine organ. Adipocytes secrete numerous factors that circulate

in blood and act on distal tissues, and they also mediate local autocrine/paracrine effects, to influence food intake, energy expenditure, and carbohydrate and lipid metabolism (1). Adiponectin (also known as apM1, AdipoQ, Gbp28, and Acrp30) is one of these adipocyte-derived factors. Epidemiological evidence has indicated that circulating adiponectin levels are reduced in patients with insulin resistance, type 2 diabetes, obesity, or cardiovascular disease (2–4). Low plasma adiponectin levels in these disease states are accompanied by reduced adiponectin gene expression in adipose tissue (5, 6). There is also evidence that adiponectin gene polymorphisms may be associated with hypoadiponectinemia, together with insulin resistance and type 2 diabetes (7). Therefore, low levels of adiponectin and adiponectin gene variation have been associated with obesity and insulin resistance.

The metabolic effects and action mechanisms for adiponectin are less clear. Adiponectin has been shown to augment lipid oxidation in skeletal muscle and myocytes (8, 9) and to reduce hepatic glucose production in liver and hepatocytes (10, 11). Accordingly, administration of adiponectin to intact rodents improved glucose tolerance and decreased plasma triglycerides (8, 9). Cell surface receptors for adiponectin have been cloned (12), and, in muscle cells, ligand binding may initiate signal transduction through phosphorylation and the activation of adenosine 5'-monophosphate-activated protein kinase (13), which is known to play a pivotal role in regulating cholesterol synthesis, lipogenesis and lipid oxidation, and glucose transport and oxidation. In addition, adiponectin may inhibit both the inflammatory process and atherogenesis by suppressing the migration of monocytes/macrophages and their transformation into foam cells in the vascular wall (14, 15).

Some insights have also been gained through work with transgenic and knockout mouse models. Recently, it was reported that overexpression of the adiponectin gene pro-

Manuscript received 28 September 2004 and in revised form 22 December 2004 and in re-revised form 17 February 2005 and in re-re-revised form 15 March 2005.

Published, JLR Papers in Press, April 16, 2005.
DOI 10.1194/jlr.M400373.JLR2005

¹ To whom correspondence should be addressed.
e-mail: yfu@uab.edu

tected ob/ob mice from diabetes and protected apolipoprotein E-deficient mice from atherosclerosis (16). Furthermore, overexpression of adiponectin in fat tissues resulted in increased circulating adiponectin levels, which in turn led to improved insulin sensitivity (17). The phenotype in adiponectin knockout mice has not been consistent, despite the fact that multiple laboratories have bred their mice into the C57BL/6 background; these mice have been reported to express baseline insulin resistance, or relative insulin resistance induced only during high-fat feeding, or no observable phenotype (18–20).

These observations have given rise to the general hypothesis that adiponectin plays a significant role in linking obesity and insulin resistance, leading ultimately to type II diabetes and atherosclerosis (21). That is, obesity-related increments in adipocyte cell size are accompanied by reduced secretion and low circulating adiponectin levels, and hypoadiponectinemia then promotes increased cellular lipid content and insulin resistance in skeletal muscle and liver. The experiments that support this hypothesis have primarily involved the cellular effects of adiponectin in muscle and liver. An understudied question in this paradigm concerns the autocrine/paracrine effects of adiponectin on adipocytes. To address this question, we established stably transfected 3T3-L1 fibroblast cell lines (22–25) using a lentiviral vector to hyperexpress adiponectin and examined the effects of adiponectin on adipocyte cell biology. We found that adiponectin promotes adipocyte differentiation and augments insulin sensitivity and lipid accumulation in mature adipocytes.

MATERIALS AND METHODS

Reagents

Mouse 3T3-L1 fibroblast cells were purchased from the American Type Culture Collection (Manassas, VA). Tissue culture media were purchased from Life Technologies (Gaithersburg, MD). Insulin, dexamethasone, and isobutylmethylxanthine were purchased from Sigma-Aldrich (St. Louis, MO). LacZ staining kit was purchased from Stratagene (San Diego, CA). RNA isolation solution was purchased from Biotecx Laboratory (Houston, TX). HRP-conjugated antibodies to the V5 epitope were purchased from Invitrogen (Carlsbad, CA). Ki67 polyclonal antibody, glucose transporter 4 (GLUT4) polyclonal antibody, and fluorescein- or rhodamine-conjugated second antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). 2-Deoxy-D-[³H]glucose and L-[1-³H]glucose were purchased from Amersham (Arlington Heights, IL). Unless otherwise specified, all other reagents were purchased from Sigma.

Recombinant lentiviruses and lentiviral transduced cell lines

Fusion cDNAs, containing the full-length adiponectin coding sequence and a V5 epitope tag, were cloned into a ViraPower-CMV vector (Invitrogen). The recombinant lentiviral plasmids and a control *LacZ* gene construct were transfected into HEK293 cells. Western blot and X-gal staining were performed to confirm that the HEK293 cell transfection was successful and infectious virus particles were produced. To establish stable 3T3-L1 cell lines that express adiponectin or *LacZ* genes, recombinant adiponectin or *LacZ* lentiviral stocks were used to infect 3T3-L1

cells with Polybrene (Specialty Media, Phillipsburg, NJ) at a final concentration of 6 $\mu\text{g}/\text{ml}$. Forty-eight hours after transduction, these cells were placed under blasticidin selection (10 $\mu\text{g}/\text{ml}$) for 20 days. Western blot analyses were performed to test for stable adiponectin or *LacZ* gene expression after antibiotic selection.

Cell culture and stimulation

3T3-L1 fibroblasts or transduced cell lines were grown and differentiated into adipocytes in 100 mm culture dishes, as described by Frost and Lane (26). Briefly, cells were grown to 100% confluence in DMEM containing 25 mM glucose and 10% calf serum at 37°C in a humidified atmosphere containing 5% CO₂. Two days after full confluence, cells were differentiated via incubation in DMEM containing 25 mM glucose, 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 10 $\mu\text{g}/\text{ml}$ insulin, and 10% FBS for 3 days and then for 2 days in DMEM containing 25 mM glucose, 10 $\mu\text{g}/\text{ml}$ insulin, and 10% FBS. Thereafter, cells were maintained in and refed every 2 or 3 days with DMEM, 25 mM glucose, and 10% FBS until used in the experiments 10–14 days after initiation of the differentiation protocol, when between 80% and 90% of the cells exhibited the adipocyte phenotype. In differentiated adipocytes, various experiments involved stimulation with insulin (10 $\mu\text{g}/\text{ml}$) for 30 min at 37°C. Conditioned media from *LacZ*-transduced or adiponectin-transduced cell lines were added with 1:1 DMEM fresh culture medium to the cell cultures.

Oil Red O staining of preadipocyte and adipocyte cells

3T3-L1 preadipocyte and adipocyte cells were stained with Oil Red O essentially as described by Ramirez-Zacarias, Castro-Munozledo, and Kuri-Harcuch (27). The 3T3-L1 cells were fixed in 10% formalin for 90 min. After washing thoroughly with distilled water, cells were incubated with a working solution of Oil Red O for 3 h (27). The staining of lipid droplets in 3T3-L1 preadipocyte and adipocyte cells was quantified using a phase-contrast microscope and Image-Pro Plus software from Media Cybernetics (Carlsbad, CA).

Triglyceride contents of 3T3-L1 preadipocyte and adipocyte cells were extracted and measured by an enzyme colorimetric assay with a kit from Sigma-Aldrich according to the manufacturer's protocol. The concentrations of cellular proteins from these cells were measured with a protein assay kit from Bio-Rad (Hercules, CA).

Glucose transport activity assays

For measurement of glucose transport activity, adipocyte cells were washed three times with transport buffer (pH 7.4), consisting of 20 mM HEPES, 120 mM NaCl, 1.2 mM MgSO₄, 2 mM CaCl₂, 2.5 mM KCl, 1 mM NaH₂PO₄, and 1 mM sodium pyruvate, and incubated in this buffer for an additional 30 min in the absence (basal) and presence of insulin (100 nM) at 37°C. Glucose transport was assayed in monolayers as initial rates of 2-deoxy glucose uptake, as described previously by our laboratory (28). In these experiments, the distribution space of radiolabeled L-glucose was used to correct for nonspecific carryover of radioactivity with the cells and uptake of hexose by simple diffusion.

GLUT4 translocation assays

Plasma membrane sheets were prepared by the method of Finigar et al. (29) with minor modifications. 3T3-L1 fibroblasts, grown on glass cover slips, were differentiated into adipocytes at least 2 weeks before the experiment. Adipocytes were then incubated for 1.75 h at 37°C in Leibovitz's L-15 medium (Life Technologies, Inc.) containing 0.2% BSA. Cover slips were incubated in the absence and presence of a maximally effective insulin concentration during the last 30 min of this incubation. Cells were then washed once with ice-cold buffer A (100 mM NaCl and 50 mM

HEPES, pH 7.3) and once with ice-cold buffer B (100 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 20 mM HEPES, pH 7.2) and then left in buffer B for sonification. Cells underwent sonification for 1 s using a Sonics and Materials VCX-400 Sonifier with a 1 inch diameter probe (model CV26) placed 1 cm above the cover slips (amplitude, 80%; pulse, 0.1 s on, 0.9 s off). Adherent plasma membrane sheets were washed twice in phosphate-buffered saline containing 0.88 mM Ca²⁺ and 0.49 mM Mg²⁺, fixed in 3% paraformaldehyde for 15 min, and processed for indirect immunofluorescence using polyclonal anti-GLUT4 antibody and FITC- or rhodamine-conjugated secondary antibodies as described (30, 31). The amounts of GLUT4 in plasma membranes were quantified by digital image processing as described previously (31). Images of the FITC-stained sheets were acquired, and the regions to be quantified were marked in the image captured with the FITC filter and counted with the Image-Pro Plus software.

Western blot analysis and immunoperoxidase cell staining

Preadipocytes and adipocytes were harvested from the culture plates with cell lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing freshly added protease inhibitor cocktail (Sigma). Twenty-five micrograms of protein per lane and known molecular weight markers from Bio-Rad were separated by SDS-PAGE. Proteins were electrophoretically transferred onto nitrocellulose membranes and incubated overnight at 4°C with blocking solution (5% nonfat milk in TBS). The blocked membranes were incubated with the HRP-conjugated V5 epitope antibody (1:1,000 dilution with 1% nonfat milk in TBS) for 1 h at room temperature and then washed three times with TBS buffer containing 0.1% Tween 20 for 15 min at room temperature with shaking. Immunodetection analyses were accomplished using the Enhance Chemiluminescence Kit (New England Nuclear Life Science Products, Boston, MA).

Immunoperoxidase cell staining followed the protocol recommended by the manufacturer (Santa Cruz Biotechnology). Briefly, cultured 3T3-L1 fibroblasts were counted and placed on sterile glass cover slips for 4 h at 37°C. These cells were washed with PBS three times and fixed with 1% formalin for 15 min. Then, after Ki67 antibody was added to the cultured cell cover slips, the ABC Staining System (Santa Cruz Biotechnology) was used to perform immunoperoxidase cell staining. The quantitative analysis of Ki67 staining was performed with Image-Pro Plus software as for the Oil Red O staining experiments described above.

Real-time quantitative PCR analysis

Approximately 5 µg of the total RNA was converted to first-strand cDNAs in 20 µl reactions using random primers (Gibco BRL). For each quantitative PCR (QPCR) experimental set, the target LDL receptor gene primers (5'-GCTTGCTGTACCTGCAAA-3' and 5'-AACTGCCGAGAGATGCACCT-3') were used to amplify 10-fold serial diluted LDL receptor gene products (10⁻¹⁰) to make a linear standard curve. For an endogenous standard, 18S rRNA primers (5'-AATTTGACTCAACACGGAAACCTCAC-3' and 5'-CAGACAAATCGCTCCACCACTAAGAAC-3') were simultaneously used for amplification from 1 µl of first-strand cDNAs in each 20 µl reaction tube; this allowed for normalization of cDNA loading in each reaction.

QPCR was performed using a Mx3000P™ Real-Time PCR System and a Brilliant SYBR Green QPCR Master Mix buffer (Stratagene) containing 100 mM KCl, 40 mM Tris-HCl, 0.4 mM of each deoxynucleoside triphosphate, 2.5 mM MgCl₂, SYBR Green I, SureStart Taq DNA polymerase (50 U/ml) with hot-start capability, and 20 nM fluorescein for experimental plate well factor collection on the Mx3000P™ Real-Time PCR System. A passive diluted reference dye (ROX) was also added to each tube (30 nM)

to compensate for non-PCR-related variations in fluorescence. The concentrations of the gene primers for adipocyte lipid binding protein (ALBP/aP2; 5'-TACTGGGCCAGGAATTTGAC-3' and 5'-GTGGAAGTGACGCCTTTTCAT-3'), peroxisome proliferator-activated receptor γ (PPARγ; 5'-TTTTCAAGGGTGCCAGTTTC-3' and 5'-AATCCTTGGCCCTCTGAGAT-3'), CCAAT/enhancer binding protein α (C/EBPα; 5'-GCTGGAGTTGACCAGTGACA-3' and 5'-AAACCATCCTCTGGGTCTCC-3'), adipocyte determination and differentiation factor 1/sterol-regulatory element binding protein 1c (ADD1/SREBP1c; 5'-GATCAAAGAGGAGCCAGTGC-3' and 5'-TAGATGGTGGCTGCTGAGTG-3'), PPARγ coactivator-1α (PGC-1α; 5'-GGCCCGCTACAGTGAGTGT-3' and 5'-GCATGAGGACTTGCTGAGTT-3'), GLUT4 (5'-GATTCTGCTGCCCTCTGTGTC-3' and 5'-ATTGGACGCTCTCTCTCCAA-3'), adiponectin receptor gene 1 (AdipoR1; 5'-TTCTTCCTCATGGCTGTGATGT-3' and 5'-AAGAAGCGCTCAGGAATTCG-3'), and AdipoR2 (5'-ATAGGGCAGATAGGCTGGTTGA-3' and 5'-GGATCCGGCAGCATACA-3') were 500 nM for each primer and 1 µl of first-strand cDNAs for each real-time QPCR performed in a volume of 20 µl. The thermal cycling program was 3 min at 95°C for enzyme activation (allowing an automated hot-start PCR), 45 cycles of denaturation for 30s at 95°C, 30 s annealing at 60°C, and 30 s extension at 72°C.

Melting curve analysis

The melting curve analysis was performed to confirm the real-time QPCR products. The amplified products were denatured and reannealed at different temperatures to detect their specific melting temperatures.

ELISA of adiponectin in cell culture media

3T3-L1 cell culture medium samples were analyzed using an Adiponectin ELISA kit from LINCO Research (St. Charles, MO). The assay was performed according to the manufacturer's protocol. Briefly, the wells of a microtiter plate coated with a pretitered amount of anti-mouse adiponectin monoclonal antibodies were loaded with 20 µl volumes of duplicate undiluted or diluted samples (depending on the cell culture stages) and adiponectin standards in the order of ascending concentration. After a second biotinylated anti-mouse polyclonal antibody was added to all of the wells, the wells were washed five times with diluted HRP buffer (50 mM Tris-buffered saline containing Tween 20). Then, 100 µl of enzyme solution (streptavidin-horseradish peroxidase) was added to each well and, after washing as above, 100 µl of substrate solution (3,3',5,5'-tetramethylbenzidine) was added to each well. The enzyme activity was measured spectrophotometrically by the increased absorbance at 450 nm, corrected from the absorbance at 590 nm, after acidification of formed products. The amount of captured adiponectin in the samples was calculated from a reference curve generated in the same assay with reference standards of known concentrations of adiponectin.

Statistics

Experimental results are shown as means ± SEM. Statistical analyses were performed by unpaired Student's *t*-test assuming unequal variance unless indicated otherwise. Significance was defined as *P* < 0.05.

RESULTS

Generation of recombinant adiponectin lentiviruses and lentiviral transduced stable cell lines

To investigate the effects of adiponectin on adipocytes, we stably transformed 3T3-L1 fibroblasts using lentiviral ex-

pression vectors. Full lengths of fusion cDNAs, including adiponectin coding sequences (0.74 kb) and a V5 epitope tag, were cloned into a ViraPower-CMV vector (Invitrogen) (Fig. 1A). The recombinant lentiviral plasmids and a control lentiviral LacZ gene construct were transfected into HEK293 cells to generate the recombinant lentiviruses. X-gal staining was performed to confirm that the HEK293 cell transfection was successful and that infectious virus particles were produced (Fig. 1B).

To establish stable 3T3-L1 fibroblast cell lines that overexpress adiponectin or LacZ genes, recombinant adiponectin or LacZ lentiviral stocks purified from HEK293 cells were used to infect 3T3-L1 fibroblasts. Forty-eight hours after transduction, these cells were placed under blasticidin selection (10 $\mu\text{g}/\text{ml}$) for 20 days. The tests for stable recombinant adiponectin or LacZ gene expression were performed after antibiotic selection by Western blot analyses (Fig. 1C).

We measured the secretion of adiponectin into cell culture media during adipocyte differentiation to confirm

adiponectin expression in the stably transduced cell lines. There was a low level of adiponectin secretion detected in the media of fibroblasts transduced with the adiponectin. However, in fully differentiated adipocytes (day 12), as shown in Fig. 1D, adiponectin levels measured by ELISA in the culture medium were 2-fold (4.19 vs. 2.21 $\mu\text{g}/\text{ml}$) above those in control cells in one of the adiponectin lentivirus transduced cell lines (Ad-18), and media adiponectin concentrations were 10-fold (22.09 vs. 2.22 $\mu\text{g}/\text{ml}$) higher in a second stably transduced cell line (Ad-23). These results were also confirmed by Western blot analyses (data not shown). All of our experiments were performed using the higher adiponectin transduced cell line (Ad-23). However, the Ad-18 cell line was also used to confirm the results obtained from the Ad-23 cell line.

Adiponectin expression in 3T3-L1 fibroblasts

In 3T3-L1 fibroblasts, we observed that cell proliferation was accelerated in adiponectin-expressing cells com-

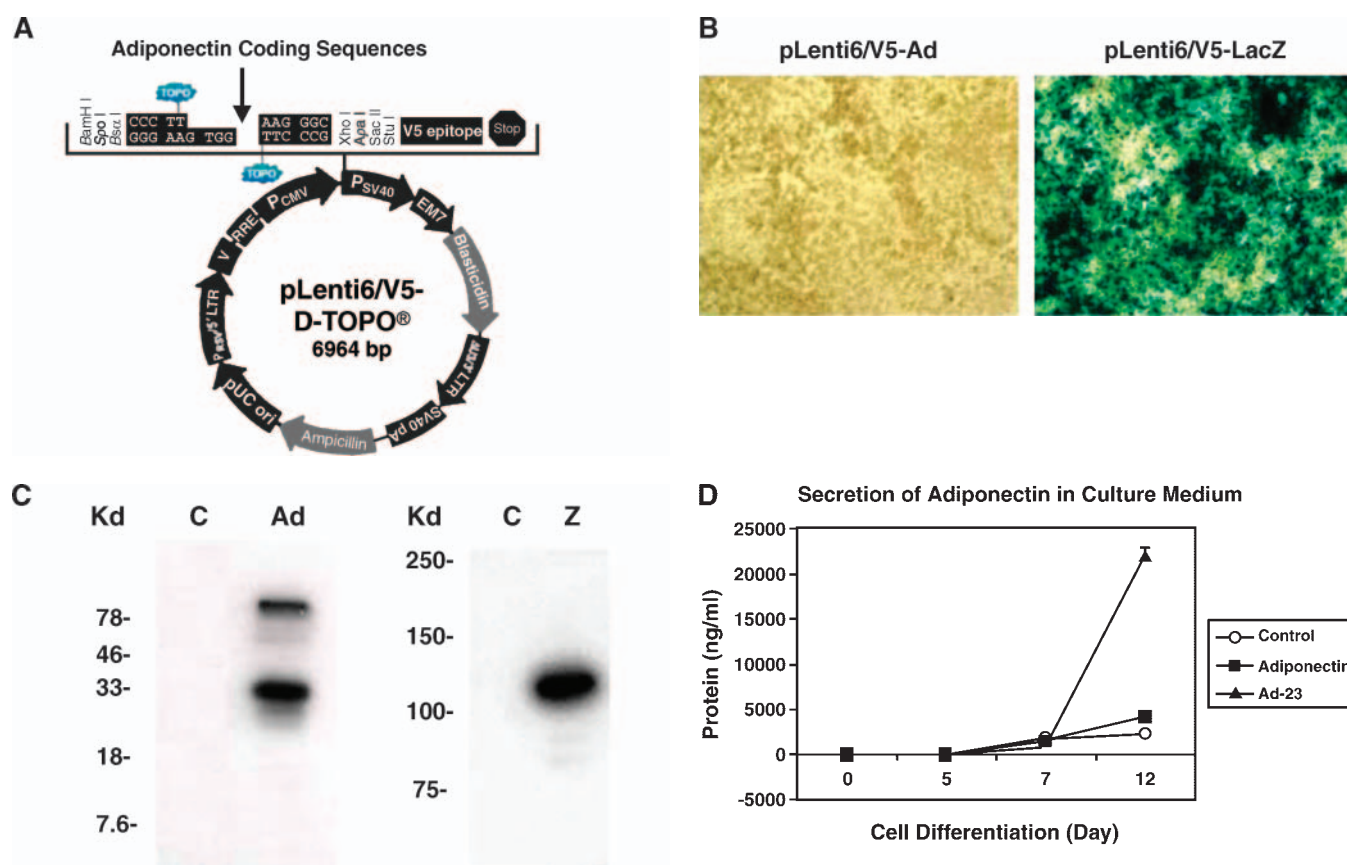


Fig. 1. Generation of recombinant adiponectin lentivirus and detection of recombinant gene expression. **A:** A full-length adiponectin coding sequence (0.74 kb) was cloned into a ViraPower-CMV vector (Invitrogen). This recombinant adiponectin gene construct included a V5 epitope tag and a CMV promoter. **B:** X-gal staining was performed on HEK293 cells transfected with recombinant lentiviral constructs containing the LacZ gene to confirm that the cell transfection was successful and infectious lentivirus particles were produced. These cells stained with X-gal indicated the presence of LacZ gene expression after 3 days of cell transfection. Recombinant adiponectin lentiviral construct-transfected cells were used as a negative control for the X-gal staining. **C:** Western blot analysis was performed to confirm the expression of the recombinant adiponectin gene (Ad) or the control LacZ gene (Z) in 3T3-L1 cells. The control lane (C) was loaded with proteins from nontransduced 3T3-L1 cells. Recombinant adiponectin and LacZ proteins were detected using anti-V5 antibodies. **D:** The cell culture media were sampled during the adipocyte differentiation process (days 0–12), and secreted adiponectin protein in these media were measured using an adiponectin ELISA. LacZ-transduced controls are indicated with open circles; one of the adiponectin-transduced cell lines (Ad-18) is represented with closed squares, and another adiponectin cell line (Ad-23) is represented with closed triangles. Results represent means \pm SEM from three separate experiments.

pared with nontransduced control cells or LacZ-transduced cells. The morphology of adiponectin-expressing fibroblast cells was unchanged; only the cell density was increased over that in the control groups before saturation density (Fig. 2A). Cell proliferation (0–72 h) was quantified at various time points before reaching full confluence by cell counting after growth arrest. Adiponectin-expressing cells exhibited increased cell number (1.38-fold to 1.78-fold) relative to control cells expressing the LacZ gene (Fig. 2B). Ki67 is a nuclear protein that is expressed in proliferating cells and has been used as a marker for active G1/S transition during cell division in solid tumors and hematological malignancies (32, 33). Therefore, we

analyzed Ki67 immunohistochemistry in both adiponectin-expressing fibroblasts and LacZ-transduced control cells that were respread at similar cell densities on sterile glass cover slips. The cell proliferation rate assessed by Ki67 staining was accelerated in the adiponectin-expressing fibroblasts (1.39-fold to 1.67-fold) compared with the controls (Fig. 2C), consistent with the observed increase in cell numbers (Fig. 2A, B).

Adiponectin hyperexpression in 3T3-L1 adipocytes

3T3-L1 fibroblasts, whether transduced with LacZ or adiponectin lentivirus, differentiated into adipocytes under the standard induction protocol (22–24). However, cells

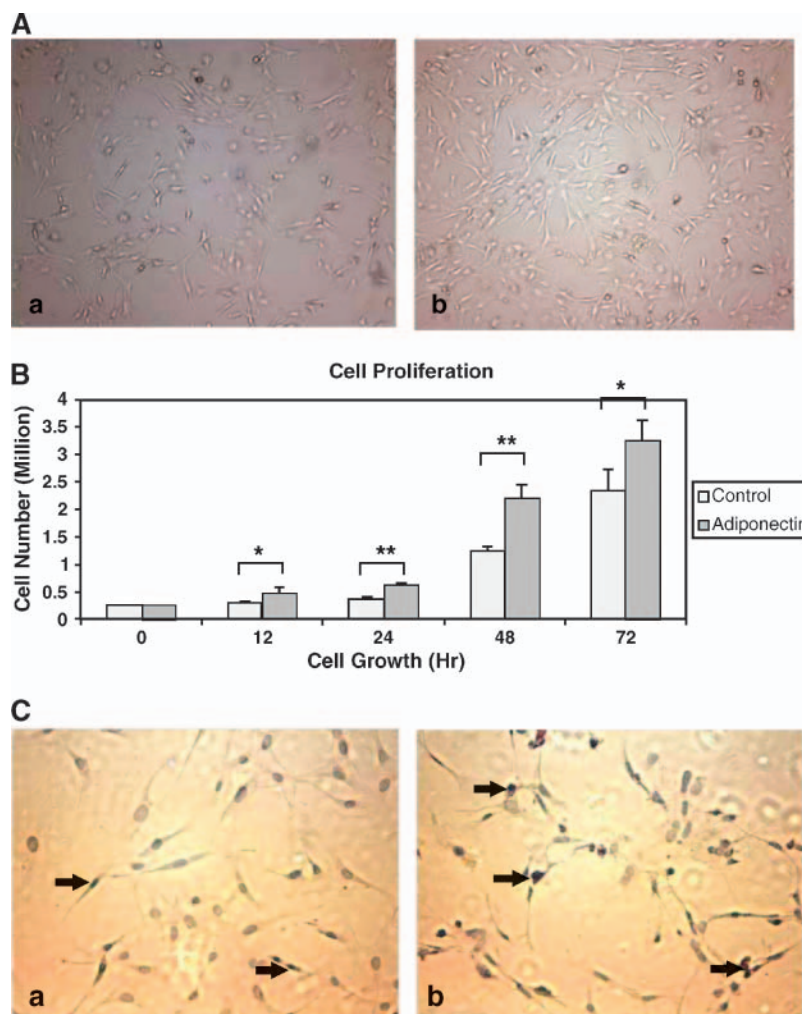


Fig. 2. Adiponectin expression accelerates the proliferation of 3T3-L1 fibroblasts. Recombinant adiponectin and LacZ lentiviruses were used to transduce 3T3-L1 fibroblasts. A: 3T3-L1 fibroblasts stably expressing LacZ (a) or adiponectin (b) genes after 12 h of cell culture. B: Adiponectin-transduced (shaded bars) and LacZ-transduced (control; open bars) 3T3-L1 fibroblasts were initially plated with 0.25 million cells at 0 h on 100 mm culture plates, and the cell numbers were subsequently counted from 12 to 72 h in cell culture before growth arrest at 100% confluence. Results represent means \pm SEM from three separate experiments. * $P < 0.05$; ** $P < 0.01$. C: Adiponectin- and LacZ-transduced 3T3-L1 fibroblasts were grown for 12 h on cell culture plates, counted, and then respread at similar densities on sterile glass cover slips for 4 h for attachment. An immunohistochemistry assay was performed with Ki67 antibodies for LacZ control (a) and adiponectin-expressing (b) fibroblasts. Arrows point to some Ki67 immunoperoxidase nuclear staining (purple in the highly condensed nuclei) in these cells, and counterstaining of cell nuclei was performed with hematoxylin (light blue). The cell numbers with Ki67 nuclear staining were 35.4% of the total cell numbers in adiponectin-overexpressed adipocytes versus 21.2% in the control cells ($P < 0.03$).

overexpressing adiponectin appeared to develop the adipocyte phenotype more rapidly. We first studied this issue by examining the time course of expression of several key transcriptional factors known to be involved in adipocyte differentiation (25), including C/EBP α , PPAR γ , and ADD1/SREBP1c. In addition, induction of ALBP/aP2 has been considered a hallmark of adipogenesis, and this is one of the target genes for PPAR γ during adipocyte differentiation (34, 35). To investigate whether the overexpression of adiponectin would modify gene expression patterns, we measured mRNAs encoding these key regulators and markers during differentiation. As shown in Fig. 3, expression of both C/EBP α and PPAR γ was more prolonged and robust over days 5 to 9 during adipocyte differentiation in adiponectin-overexpressing cells compared with LacZ-transduced controls. Accordingly, ALBP/aP2 expression was markedly increased in the adiponectin-overexpressing cells over this time period. ADD1/SREBP1c gene expression was also significantly augmented during the later phases of differentiation in the adiponectin-overexpressing adipocytes.

The PGC-1 α pathway plays a crucial role in the transcriptional regulation of hepatic gluconeogenic enzymes (36, 37) and in the differentiation of brown fat cells. In LacZ controls, PGC-1 α expression was high in fibroblasts and then was suppressed to low levels during adipogenesis (Fig. 3E). Interestingly, transduction with the recombinant adiponectin lentivirus led to the suppression of PGC-1 α in fibroblasts and to a greater degree of suppression during early differentiation, relative to LacZ controls at comparable stages of differentiation. However, as differentiation progressed, PGC-1 α mRNA copy numbers were similarly suppressed to very low levels between days 5 and 12 in both adiponectin-overexpressing cells and in LacZ-transduced controls (Fig. 3E).

We also studied AdipoR1 and AdipoR2 and determined that both genes are expressed in 3T3-L1 adipocytes. Interestingly, both of the adiponectin receptor genes showed lower levels of expression in adiponectin-overexpressing adipocytes compared with LacZ control cells (Fig. 3F, G).

Effects of adiponectin hyperexpression on the 3T3-L1 adipocyte phenotype

Oil Red O staining was performed and histologically quantified to assess the effects on cellular lipid content. Compared with lentiviral LacZ-transduced adipocytes, adiponectin-transduced adipocytes accumulated lipid at a more rapid rate during adipogenesis, and in fully differentiated cells they contained greater amounts of lipid with larger cytoplasmic lipid droplets (Fig. 4A–D). At day 12 of differentiation, the mean proportion of cell area stained with Oil Red O was more than 4-fold greater in adiponectin-overexpressed adipocytes (12.6% of area) compared with that in LacZ control cells (3.04% of area; $P < 0.05$). Cellular triglycerides from these cells were also measured to confirm the lipid accumulation (Fig. 4E). When the conditioned medium obtained from adiponectin-transduced cell cultures was added to the control LacZ-transduced cell cultures, a significantly increased cellular triglyceride ($P < 0.05$) was also observed compared with the LacZ conditioned medium cultured cells (Fig. 4F).

Because adiponectin overexpression led to an increase in lipid accumulation, we hypothesized that these cells would also display an increase in insulin-stimulated glucose transport. As shown by the data in Fig. 5, there was a 1.8-fold increase ($P < 0.01$) in the insulin-stimulated glucose uptake in fully differentiated (day 12 cells) adiponectin-overexpressing adipocytes (bar 5, Adiponectin + Insulin) compared with control adipocytes (bar 2, Control + Insulin). When control cells (LacZ-transduced) were incubated with the conditioned medium from adiponectin-transduced adipocytes [bar 6, Ad(m) + Insulin], insulin-stimulated glucose uptake was increased significantly ($P < 0.01$) compared with that in control cells treated with conditioned medium from the LacZ-transduced adipocytes [bar 3, Control(m) + Insulin]. Full insulin dose-response curves indicated that glucose transport rates were increased in the adiponectin-overexpressing cells at all insulin concentrations, such that there were no significant changes in insulin half maximal effective dose (ED50) for glucose transport stimulation (data not shown).

To determine the underlying mechanism, we assessed insulin's ability to recruit GLUT4 proteins to the cell surface (38) using the plasma membrane "lawn" assay. As seen in Fig. 6B, D, plasma membrane-associated GLUT4 after insulin stimulation was clearly increased in the adiponectin-overexpressing adipocytes compared with control LacZ-transduced adipocytes; the mean areas of cell membrane with immunofluorescence were 10.73% in adiponectin-overexpressed adipocytes versus 3.77% in the control cells ($P < 0.05$). However, without insulin stimulation, we observed no significant differences in the amount of plasma membrane GLUT4 compared with basal cells (Fig. 6A, C). To identify whether increased recruitment of GLUT4 to the plasma membrane could be associated with an increase in GLUT4 gene expression, we measured GLUT 4 mRNA in both adiponectin-overexpressing and control LacZ adipocytes. Figure 7 shows that GLUT4 mRNA levels were not affected by adiponectin from 0 to 9 days of differentiation, but by day 12, they were increased by 81% in adiponectin-overexpressing adipocytes.

DISCUSSION

Adiponectin is one of multiple adipocytokines secreted by adipose tissue and has been shown to modulate both glucose and lipid metabolism in vivo and in vitro. For the most part, these studies have examined the metabolic effects of adiponectin in muscle and liver. Whether adiponectin acts as an autocrine/paracrine factor to regulate adipocyte biology has not been rigorously examined. The current studies for the first time demonstrate that overexpression of adiponectin can enhance 3T3-L1 fibroblast proliferation, accelerate adipocyte differentiation, and, in fully differentiated adipocytes, augment both lipid accumulation and insulin-responsive glucose transport. These effects were observed in stably transduced cell lines exhibiting both moderate (2 times that in controls) and high

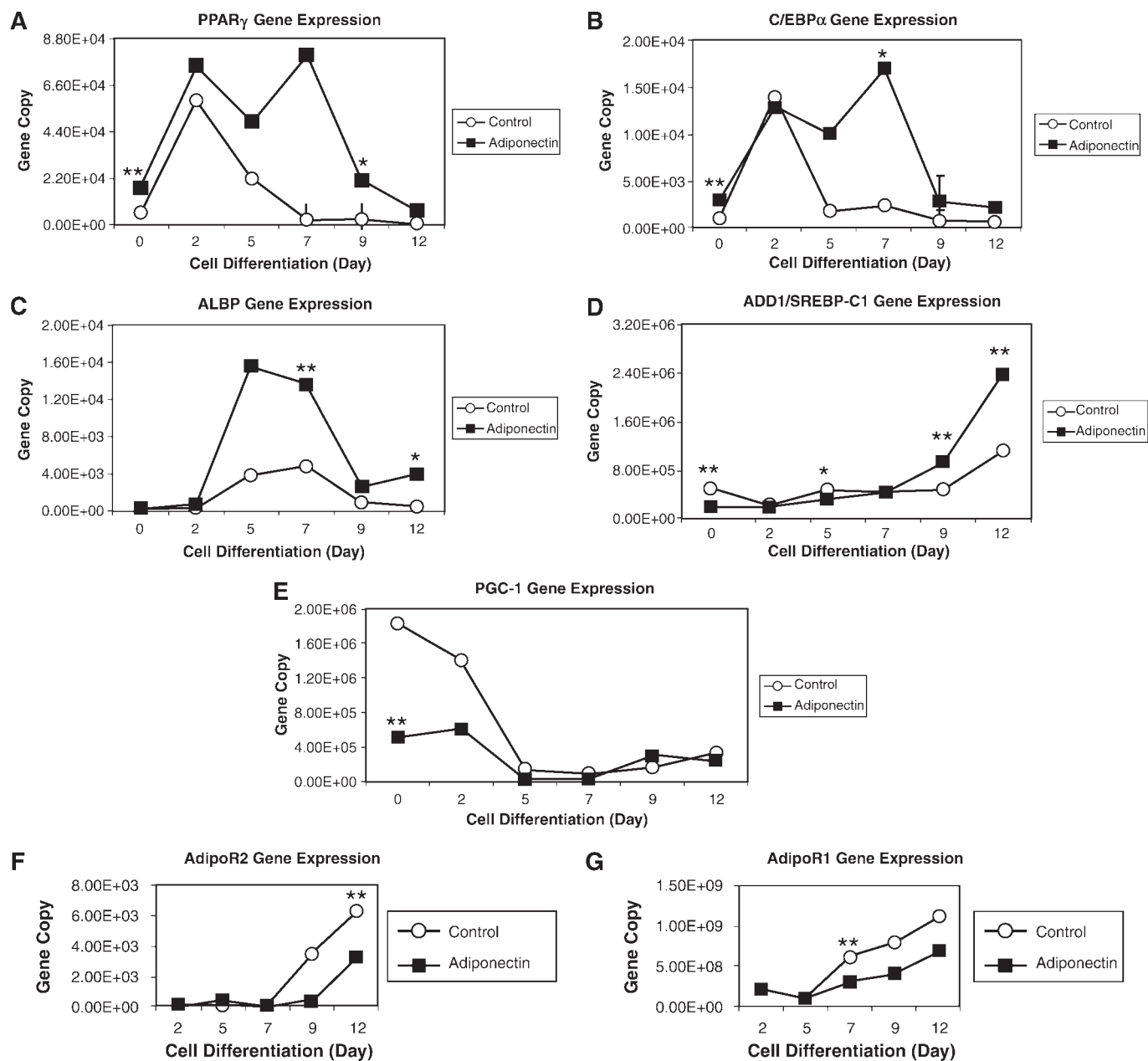


Fig. 3. Effects of adiponectin on gene expression patterns during adipogenesis. Day 0 represents 3T3-L1 fibroblasts reaching 100% confluence. Two days after full confluence (day 2), cells were placed in DMEM containing 25 mM glucose, 0.5 mM isobutylmethylxanthine, 1 μ M dexamethasone, 10 μ g/ml insulin, and 10% FBS for 3 days; then on day 5, cells were placed in DMEM containing 25 mM glucose, 10 μ g/ml insulin, and 10% FBS for 2 days. After day 7, cells were maintained in DMEM, 25 mM glucose, and 10% FBS. At the indicated time points, adiponectin- and LacZ-transduced preadipocytes and adipocytes were lysed and the mRNA levels encoding peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT/enhancer binding protein α (C/EBP α), adipocyte determination and differentiation factor 1/sterol-regulatory element binding protein 1c (ADD1/SREBP1c), adipocyte lipid binding protein (ALBP/aP2), PPAR γ coactivator-1 α (PGC-1 α), and adiponectin receptor genes 1 and 2 (AdipoR1 and AdipoR2) were quantified by quantitative PRC. Results represent means \pm SEM from three separate experiments. * $P < 0.05$; ** $P < 0.01$.

(10 times that in controls) levels of adiponectin overexpression compared with untransduced control cells or control cells stably transduced with the LacZ gene. Consistent with our results, others have also reported that overexpression of the adiponectin gene in fat tissue results in more lipid accumulation in the adipocytes (17).

These effects in adipocytes are somewhat unexpected based on epidemiological data and suggest a new role for

adiponectin in adipocyte biology. Several clinical studies have observed that circulating adiponectin levels are reduced in obesity, in which mean adipocyte size is increased, and increased in lean individuals with smaller adipocyte size. On the surface, this seems to contradict our results showing that adiponectin overexpression accelerates adipogenesis and augments cellular lipid accumulation. These observations can be reconciled by our findings

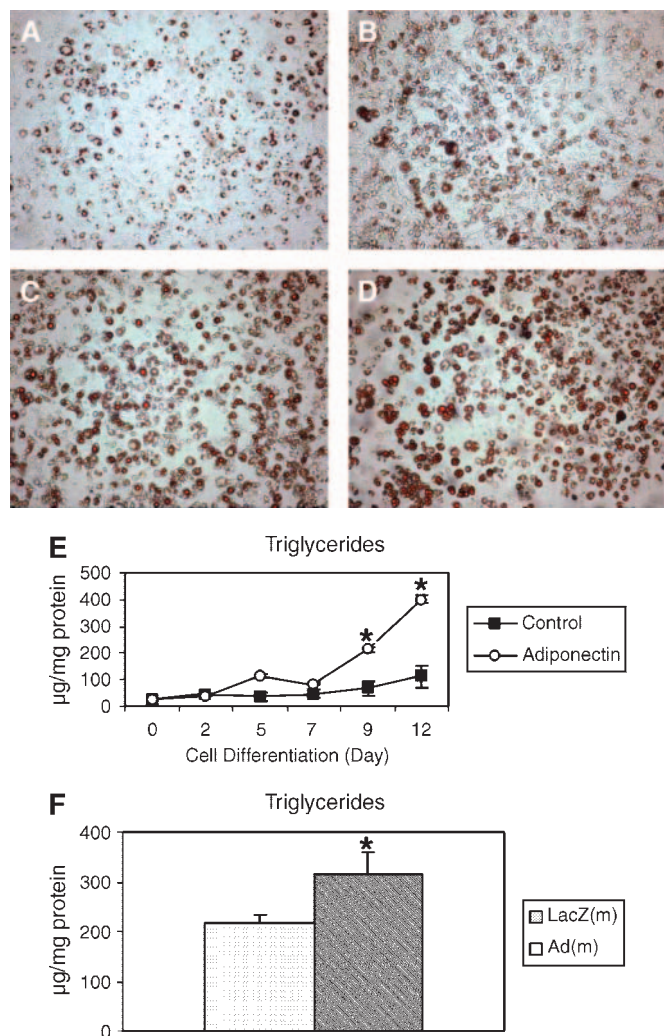


Fig. 4. Effects of adiponectin on lipid droplet accumulation in adipocytes. Oil Red O staining was performed to examine the content of lipid in adipocytes. A: Lentiviral LacZ-transduced adipocytes at day 7 of differentiation. B: Adiponectin-transduced adipocytes at day 7 of differentiation. C: Lentiviral LacZ-transduced adipocytes at day 12 of differentiation. D: Adiponectin-transduced adipocytes at day 12 of differentiation. Adiponectin-overexpressing adipocytes showed more lipid accumulation than the control cells (stained cell area, 12.35% vs. 3.04%; $P < 0.05$). E: Cellular triglycerides were measured by a colorimetric assay during cell cultures. F: Cellular triglycerides were also measured by a colorimetric assay in conditioned medium-treated cell cultures at day 12. Ad(m), adiponectin-conditioned medium; LacZ(m), LacZ-conditioned medium. Results represent means \pm SEM from three separate experiments. * $P < 0.05$.

that adiponectin functions as an adipocyte differentiation factor. We propose that adiponectin acts locally at the tissue level to maintain adipocyte size and mass around an equilibrium set point. After weight loss, smaller adipocytes secrete more adiponectin, which has the effect of promoting adipocyte differentiation and lipid accumulation, thus returning adipocytes to their baseline size. Conversely, with weight gain, the reduction in adiponectin secretion leads to decelerated lipid accumulation and a reduction in adipocyte size to the baseline level. Such a mechanism exists for leptin, another adipocyte protein for which

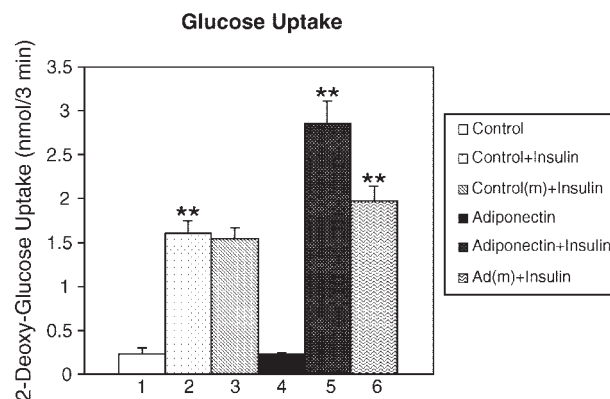


Fig. 5. Adiponectin and conditioned medium containing adiponectin enhance insulin-stimulated glucose transport. Adipocytes transduced with the LacZ gene (Control) or with the human adiponectin gene (Adiponectin) were incubated for 12 days until fully differentiated. Control cells (bars 1 and 2) and cells overexpressing adiponectin (bars 4 and 5) were incubated in the absence (bars 1 and 4) or presence (bars 2 and 5) of insulin for 30 min at 37°C to induce maximal rates of glucose uptake. Control cells were also incubated either with conditioned medium from the cultures of control adipocytes transduced with the LacZ gene (bar 3) or with conditioned medium from cells transduced with the adiponectin gene (bar 6) in the presence of insulin. Ad(m) refers to control cells to which conditioned medium from the adiponectin-transduced cells was added, and Control(m) refers to control cells to which conditioned medium from the LacZ-transduced cells was added. The rate of insulin-stimulated glucose transport was then determined. Results represent means \pm SEM from three separate experiments. ** $P < 0.01$ for Adiponectin + Insulin versus Control + Insulin and for Ad(m) + Insulin versus Control(m) + Insulin.

secretory rates vary as a function of cell size, although leptin modulates adipocyte size indirectly via effects on appetite centers in the central nervous system. In a complementary manner, adiponectin could help to maintain equilibrium adipocyte size via metabolic effects as an autocrine/paracrine factor in adipose tissue. However, Yokota et al. (39) have reported that adding adiponectin to cloned stromal preadipocyte can inhibit the differentiation of these cells into fat cells, although they also observed no such inhibition in 3T3-L1 adipocytes. Probably, adiponectin can play different roles in different types of cells.

3T3-L1 fibroblasts stably transduced with adiponectin lentivirus exhibited a rapid growth phase and reached cell contact inhibition more quickly than in the control cells. During cell proliferation, a key checkpoint in the active cell cycles is the expression of Ki67 nuclear protein, which is a marker for the G1/S transition (40). The G1/S transition is mediated by two main pathways (cyclin D1/p16INK4A/pRb and p14ARF/p53/MDM2), and the activities of these pathways are correlated with the amounts of Ki67 nuclear protein in cells. In tumors and some hematological malignancies, the higher proliferation rate is indicated by a greater proportion of cells staining for Ki67; on the other hand, blockage of the G1/S transition causing cell cycle arrest and a reduction in the proliferation rate results in a marked diminution of Ki67 (41). In similar numbers of control and adiponectin lentivirus-transduced fibroblasts,

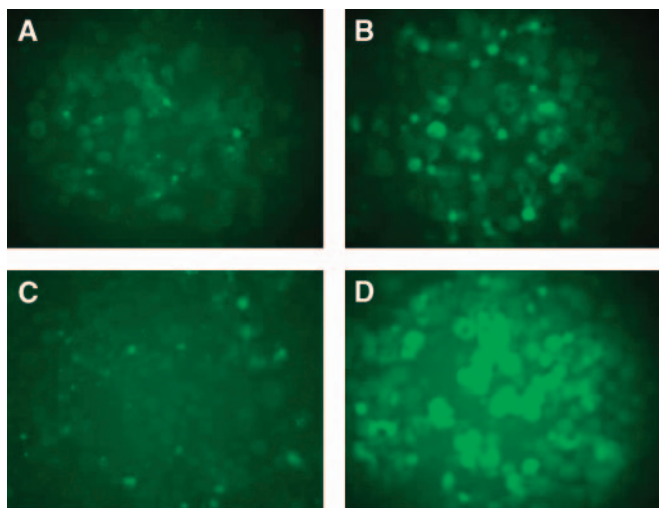


Fig. 6. Effects of adiponectin on insulin-mediated recruitment of glucose transporter 4 (GLUT4) to the plasma membrane. Adiponectin-transduced 3T3-L1 fibroblasts and control LacZ-transduced fibroblasts were grown on glass cover slips and differentiated into adipocytes. Adipocytes were then stimulated for 30 min without (basal) or with 100 nM insulin. The adipocytes were then washed and disrupted by sonication, leaving the plasma membrane sheets attached to cover slips (the plasma membrane “lawn” assay). Plasma membrane-associated GLUT4 was detected using a polyclonal anti-GLUT4 antibody and a FITC-conjugated secondary antibody. A: Basal LacZ-expressing adipocytes. B: Insulin-stimulated LacZ-expressing adipocytes. C: Basal adiponectin-hyperexpressing adipocytes. D: Insulin-stimulated adiponectin-hyperexpressing adipocytes.

the Ki67 marker was detected to a much greater extent in adiponectin-expressing cells compared with LacZ controls, indicating that adiponectin accelerates cell growth by increasing the number of cells undergoing the G1/S transition. Whether this increased cell proliferation is related to a relative suppression of PGC-1 α in adiponectin-transduced cells remains to be determined.

Adipogenesis from fibroblast precursors follows a highly ordered and well-characterized temporal sequence. The first step is the growth arrest of proliferating preadipocytes. In 3T3-L1 preadipocytes, initial growth arrest is in-

duced by the addition of a prodifferentiative hormonal regimen. This is followed by the coordinated sequential expression of key transcription factors that direct the adipogenic program. It has been proven that PPAR γ and C/EBP α are two of the key transcriptional factors for initiating adipocyte differentiation (42–47) and that they positively regulate each other’s expression. However, PPAR γ is felt to play a directorial role in the adipogenic hierarchy of transcription factors, whereas C/EBP α promotes specific aspects of the adipocyte phenotype, including insulin sensitivity and lipid accumulation (25). Concomitant with accelerated differentiation, adiponectin overexpression led to a more marked and prolonged expression of PPAR γ and C/EBP α during adipogenesis. An additional role for ADD1/SREBP1c was suggested by the observation that this gene was induced dramatically as cultured preadipocytes were stimulated to undergo differentiation (48). Overexpression of the ADD1/SREBP1c gene in 3T3-L1 cells, in the presence of hormonal inducers of differentiation, augments the expression of adipocyte marker genes as well as lipid accumulation, analogous with the current results for adiponectin. Also, expression of a dominant-negative ADD1/SREBP1c (a non-DNA binding mutant targeted to the nucleus) completely abolishes the ability of preadipocytes to undergo differentiation (49). These results are consistent with a required role for ADD1/SREBP1c in adipogenesis, but, unlike PPAR γ , ADD1/SREBP1c cannot initiate the adipogenic process per se. We observed that ADD1/SREBP1c gene expression was also increased in adiponectin-overexpressing cells in the later stages of adipogenesis. Thus, the acceleration of adipogenesis in adiponectin-overexpressing cells was accompanied by a pronounced salutary effect on key transcription factors that direct the differentiation paradigm.

PPAR γ and C/EBP α direct the expression of a multiplicity of genes that are responsible for the fully differentiated adipocyte phenotype, and two key functional proteins are ALBP/aP2 and GLUT4 (47). With the expression of these proteins, cells acquire a capacity for insulin-stimulated glucose transport, and lipid droplets begin to appear in the cytoplasm and over time coalesce into fewer major

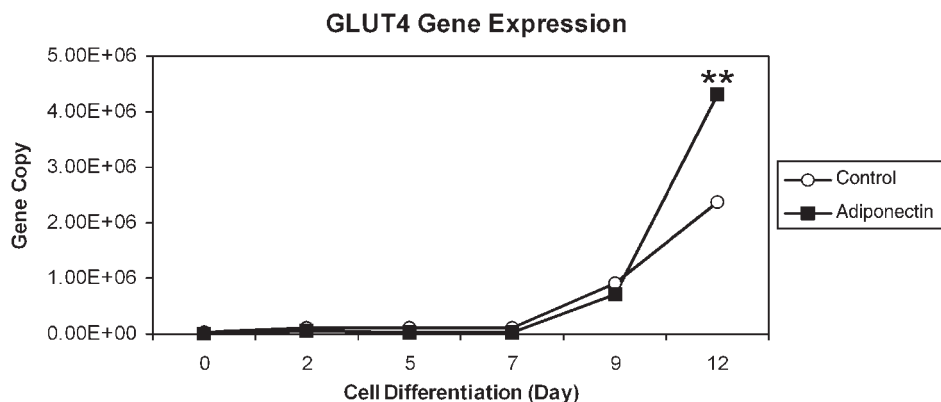



Fig. 7. Adiponectin increases the gene expression of GLUT4. Expression of the GLUT4 gene was examined using quantitative PCR for adiponectin-transduced adipocytes and LacZ-transduced adipocytes during the differentiation process. Results represent means \pm SEM from three separate experiments. ** $P < 0.01$.

droplets in the cells. Both of these proteins were induced in overabundance in the adiponectin lentivirus-transduced cells. Increased expression of these proteins at least partly explained why these adipocytes exhibited enhanced insulin responsiveness for the stimulation of glucose transport and increased lipid content. GLUT4 proteins reside mostly in intracellular microsomal membranes in unstimulated 3T3-L1 adipocytes and are acutely translocated to the cell surface in response to insulin (38). Our studies indicate that the mechanism underlying the increase in insulin sensitivity is that adiponectin increases total GLUT4 expression and the numbers of GLUT4 transporters acutely recruited to the plasma membrane in response to insulin. When we used the conditioned medium from the adiponectin-transduced cell cultures to treat 3T3-L1 adipocytes, the same results were observed for the increased GLUT4 activity in these treated cells (Fig. 5), even though GLUT4 activity was not increased to the same level as that from the adiponectin-transduced cells. One of the reasons for this difference is probably the different levels of adiponectin in the media and the cells.

PGC-1 α was cloned from brown adipose tissue (50), but it is not expressed in white adipose tissue. When expressed ectopically in white fat cells in vitro, PGC-1 α induces genes that are associated with the brown fat cell phenotype, including UCP-1 and some components of the electron transport chain (51). In both adiponectin lentivirus-transduced and control LacZ-transduced cells, PGC-1 α mRNA was detected in 3T3-L1 fibroblasts, and the levels were progressively suppressed to very low levels during the course of adipocyte differentiation. However, in adiponectin-expressing fibroblasts, PGC-1 α mRNA levels were reduced by ~50% compared with controls. Thus, even the low levels of adiponectin secretion observed in lentivirus-transduced fibroblasts appeared to affect gene expression.

Two adiponectin receptors (AdipoR1 and AdipoR2) have been cloned from skeletal muscle and liver and mediate positive effects on lipid and glucose metabolism in these tissues through signal transduction pathways involving AMP kinase and PPAR α (12, 13). The expression of two adiponectin receptors has also been reported in adipocytes and macrophages (52, 53). As we have observed from our data, these two adiponectin receptors were downregulated or desensitized in the adiponectin-overexpressing adipocytes, probably as a result of the feedback effects of more adiponectin expression and lipid accumulation in the adiponectin-transduced adipocytes. These results support the contention that adiponectin acts as an autocrine/paracrine factor in vivo and could participate in the regulation of adipocyte metabolism and adipose tissue mass. 

The authors are grateful to Dr. Ling Li for kindly helping in the measurement of lipid droplets and Dr. Chandrika Piyathilake for her suggestions regarding Ki67 experiments. The authors thank Jennifer Jones for assisting in the immunoperoxidase staining experiments. This work was supported by grants from the National Institutes of Health (DK-38764 to W.T.G.; PO1-HL-55782 to W.T.G. and R.L.K.) and by the Merit Review Program of the Department of Veterans Affairs (to W.T.G. and R.L.K.).

REFERENCES

- Havel, P. 2002. Control of energy homeostasis and insulin action by adipocyte hormones: leptin, acylation stimulating protein, and adiponectin. *Curr. Opin. Lipidol.* **13**: 51–59.
- Arita, Y., S. Kihara, N. Ouchi, M. Takahashi, K. Maeda, J. Miyagawa, K. Hotta, I. Shimomura, T. Nakamura, K. Miyaoka, et al. 1999. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem. Biophys. Res. Commun.* **257**: 79–83.
- Lindsay, R. S., T. Funahashi, R. L. Hanson, Y. Matsuzawa, S. Tanaka, P. A. Tataranni, W. C. Knowler, and J. Krakoff. 2002. Adiponectin and development of type 2 diabetes in the Pima Indian population. *Lancet.* **360**: 57–58.
- Lindsay, R. S., T. Funahashi, J. Krakoff, Y. Matsuzawa, S. Tanaka, S. Kobes, P. H. Bennett, P. A. Tataranni, W. C. Knowler, and R. L. Hanson. 2003. Genome-wide linkage analysis of serum adiponectin in the Pima Indian population. *Diabetes.* **52**: 2419–2425.
- Hotta, K., T. Funahashi, N. L. Bodkin, H. K. Ortmeier, Y. Arita, B. C. Hansen, and Y. Matsuzawa. 2001. Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. *Diabetes.* **50**: 1126–1133.
- Weyer, C., T. Funahashi, S. Tanaka, K. Hotta, Y. Matsuzawa, R. E. Pratley, and P. A. Tataranni. 2001. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J. Clin. Endocrinol. Metab.* **86**: 1930–1935.
- Waki, H., T. Yamauchi, J. Kamon, Y. Ito, S. Uchida, S. Kita, K. Hara, Y. Hada, F. Vasseur, P. Froguel, et al. 2003. Impaired multimerization of human adiponectin mutants associated with diabetes: molecular structure and multimer formation of adiponectin. *J. Biol. Chem.* **278**: 40352–40363.
- Fruebis, J., T. S. Tsao, S. Javorschi, D. Ebbets-Reed, M. R. Erickson, F. T. Yen, B. E. Bihain, and H. F. Lodish. 2001. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc. Natl. Acad. Sci. USA.* **98**: 2005–2010.
- Yamauchi, T., J. Kamon, H. Waki, Y. Terauchi, N. Kubota, K. Hara, Y. Mori, T. Ide, K. Murakami, N. Tsuboyama-Kasaoka, et al. 2001. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipodystrophy and obesity. *Nat. Med.* **7**: 941–946.
- Berg, A. H., T. P. Combs, X. Du, M. Brownlee, and P. E. Scherer. 2001. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat. Med.* **7**: 947–953.
- Combs, T. P., A. H. Berg, S. Obici, P. E. Scherer, and L. Rossetti. 2001. Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. *J. Clin. Invest.* **108**: 1875–1881.
- Yamauchi, T., J. Kamon, Y. Ito, A. Tsuchida, T. Yokomizo, S. Kita, T. Sugiyama, M. Miyagishi, K. Hara, M. Tsunoda, et al. 2003. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature.* **423**: 762–768.
- Yamauchi, T., J. Kamon, Y. Minokoshi, Y. Ito, H. Waki, S. Uchida, S. Yamashita, M. Noda, S. Kita, K. Ueki, et al. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat. Med.* **8**: 1288–1295.
- Ouchi, N., S. Kihara, Y. Arita, Y. Okamoto, K. Maeda, H. Kuriyama, K. Hotta, M. Nishida, M. Takahashi, M. Muraguchi, et al. 2000. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF- κ B signaling through a cAMP-dependent pathway. *Circulation.* **102**: 1296–1301.
- Ouchi, N., S. Kihara, Y. Arita, M. Nishida, A. Matsuyama, Y. Okamoto, M. Ishigami, H. Kuriyama, K. Kishida, H. Nishizawa, et al. 2001. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation.* **103**: 1057–1063.
- Yamauchi, T., J. Kamon, H. Waki, Y. Imai, N. Shimosawa, K. Hioki, S. Uchida, Y. Ito, K. Takakuwa, J. Matsui, et al. 2003. Globular adiponectin protected ob/ob mice from diabetes and apoE-deficient mice from atherosclerosis. *J. Biol. Chem.* **278**: 2461–2468.
- Combs, T. P., U. B. Pajvani, A. H. Berg, Y. Lin, L. A. Jelicks, M. Laplante, A. R. Nawrocki, M. W. Rajala, A. F. Parlow, L. Cheeseboro, et al. 2004. A transgenic mouse with a deletion in the collagenous domain of adiponectin displays elevated circulating adiponectin and improved insulin sensitivity. *Endocrinology.* **145**: 367–383.
- Kubota, N., Y. Terauchi, T. Yamauchi, T. Kubota, M. Moroi, J. Matsui, K. Eto, T. Yamashita, J. Kamon, H. Satoh, et al. 2002. Disruption of adiponectin causes insulin resistance and neointimal formation. *J. Biol. Chem.* **277**: 25863–25866.

19. Maeda, N., I. Shimomura, K. Kishida, H. Nishizawa, M. Matsuda, H. Nagaretani, N. Furuyama, H. Kondo, M. Takahashi, Y. Arita, et al. 2002. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat. Med.* **8**: 731–737.
20. Ma, K., A. Cabrero, P. K. Saha, H. Kojima, L. Li, B. H.-J. Chang, A. Paul, and L. Chan. 2002. Increased β -oxidation but no insulin resistance or glucose intolerance in mice lacking adiponectin. *J. Biol. Chem.* **277**: 34658–34661.
21. Heilbronm, L. K., S. R. Smith, and E. Ravussin. 2003. The insulin-sensitizing role of the fat derived hormone adiponectin. *Curr. Pharm. Des.* **9**: 1411–1418.
22. Green, H., and O. Kehinde. 1974. Sublines of mouse 3T3 cells that accumulate lipid. *Cell.* **1**: 113–116.
23. Green, H., and O. Kehinde. 1975. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell.* **5**: 19–27.
24. Green, H., and O. Kehinde. 1976. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell.* **7**: 105–113.
25. Rosen, E. D., C. J. Walkey, P. Puigserver, and B. M. Spiegelman. 2000. Transcriptional regulation of adipogenesis. *Genes Dev.* **14**: 1293–1307.
26. Frost, S., and M. Lane. 1985. Evidence for the involvement of vicinal sulfhydryl groups in insulin-activated hexose transport by 3T3-L1 adipocytes. *J. Biol. Chem.* **260**: 2646–2652.
27. Ramirez-Zacarias, J. L., F. Castro-Munozledo, and W. Kuri-Harcuch. 1992. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil Red O. *Histochemistry.* **97**: 493–497.
28. Mayor, P., L. Maianu, and W. T. Garvey. 1992. Glucose and insulin chronically regulate insulin action via different mechanisms in BC3H1 myocytes: effects on glucose transporter gene expression. *Diabetes.* **41**: 274–285.
29. Fingar, D. C., S. Hausdorff, J. Blenis, and M. J. Birnbaum. 1993. Dissociation of pp70 ribosomal protein S6 kinase from insulin-stimulated glucose transport in 3T3-L1 adipocytes. *J. Biol. Chem.* **268**: 3005–3008.
30. Robinson, L., S. Pang, D. Harris, J. Heuser, and D. E. James. 1992. Translocation of the glucose transporter (GLUT4) to the cell surface in permeabilized 3T3-L1 adipocytes: effects of ATP insulin, and GTP gamma S and localization of GLUT4 to clathrin lattices. *J. Cell Biol.* **117**: 1181–1196.
31. Nelson, B. A., K. A. Robinson, and M. G. Buse. 2000. High glucose and glucosamine induce insulin resistance via different mechanisms in 3T3-L1 adipocytes. *Diabetes.* **49**: 981–991.
32. Gore, S. D., L. J. Weng, and P. J. Burke. 1993. Validation of flow-cytometric determination of Ki67 expression as a measure of growth factor response in acute myelogenous leukemia. *Exp. Hematol.* **21**: 1702–1708.
33. Lopez, F., F. Belloc, F. Lacombe, P. Dumain, J. Reiffers, P. Bernard, and M. R. Boisseau. 1991. Modalities of synthesis of Ki67 antigen during the stimulation of lymphocytes. *Cytometry.* **12**: 42–49.
34. Tontonoz, P., E. Hu, R. A. Graves, A. I. Budavari, and B. M. Spiegelman. 1994. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev.* **8**: 1224–1234.
35. Rosen, E. D., P. Scarraf, A. E. Troy, G. Bradwin, K. Moore, D. S. Milstone, B. M. Spiegelman, and R. M. Mortensen. 1999. PPAR γ is required for the differentiation of adipose tissue in vivo and in vitro. *Mol. Cell.* **4**: 611–617.
36. Yoon, J. C., P. Puigserver, G. Chen, J. Donovan, Z. Wu, J. Rhee, G. Adelmant, J. Stafford, C. R. Kahn, D. K. Granner, et al. 2001. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature.* **413**: 131–138.
37. Yoon, J. C., G. Xu, J. T. Deeney, S.-N. Yang, J. Rhee, P. Puigserver, A. R. Levens, R. Yang, C.-Y. Zhang, B. B. Lowell, et al. 2003. Suppression of β cell energy metabolism and insulin release by PGC-1 α . *Dev. Cell.* **5**: 73–83.
38. Hunter, S., and W. T. Garvey. 1998. Insulin action and insulin resistance: diseases involving defects in insulin receptors, signal transduction, and the glucose transport effector system. *Am. J. Med.* **105**: 331–345.
39. Yokota, T., C. S. Meka, K. L. Medina, H. Igarashi, P. C. Comp, M. Takahashi, M. Nishida, K. Oritani, J. Miyagawa, T. Funahashi, et al. 2002. Paracrine regulation of fat cell formation in bone marrow cultures via adiponectin and prostaglandins. *J. Clin. Invest.* **109**: 1303–1310.
40. Antonescu, C. R., D. H. Leung, M. Dudas, M. Ladanyi, M. Brennan, J. M. Woodruff, and C. Cordon-Cardo. 2000. Alterations of cell cycle regulators in localized synovial sarcoma: a multifactorial study with prognostic implications. *Am. J. Pathol.* **156**: 977–983.
41. Manjeshwar, S., D. E. Branam, M. R. Lerner, D. J. Brackett, and E. R. Jupe. 2003. Tumor suppression by the prohibitin gene 3' untranslated region RNA in human breast cancer. *Cancer Res.* **63**: 5251–5256.
42. Morrison, R. F., and S. R. Farmer. 1999. Role of PPAR in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18 (INK4c) and p21 (Waf1/Cip1), during adipogenesis. *J. Biol. Chem.* **274**: 17088–17097.
43. Shao, D., and M. A. Lazar. 1997. Peroxisome proliferator activated receptor γ , CCAAT/enhancer-binding protein α , and cell cycle status regulate the commitment to adipocyte differentiation. *J. Biol. Chem.* **272**: 21473–21478.
44. Freytag, S. O., D. L. Paielli, and J. D. Gilbert. 1994. Ectopic expression of the CCAAT/enhancer-binding protein alpha promotes the adipogenic program in a variety of mouse fibroblastic cells. *Genes Dev.* **8**: 1654–1663.
45. Lin, F. T., and M. D. Lane. 1994. CCAAT/enhancer binding protein α is sufficient to initiate the 3T3-L1 adipocyte differentiation program. *Proc. Natl. Acad. Sci. USA.* **91**: 8757–8761.
46. Wang, N. D., M. J. Finegold, A. Bradley, C. N. Ou, S. V. Abdelsayed, M. D. Wilde, L. R. Taylor, D. R. Wilson, and G. J. Darlington. 1995. Impaired energy homeostasis in C/EBP-alpha knockout mice. *Science.* **269**: 1108–1112.
47. Spiegelman, B. M., L. Choy, G. Hotamisligil, R. A. Graves, and P. Tontonoz. 1993. Regulation of adipocyte gene expression in differentiation and syndromes of obesity/diabetes. *J. Biol. Chem.* **268**: 6823–6826.
48. Kim, J. B., and B. M. Spiegelman. 1996. ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes Dev.* **10**: 1096–1107.
49. Shimomura, I., R. E. Hammer, J. A. Richardson, S. Ikemoto, Y. Bashmakov, J. L. Goldstein, and M. S. Brown. 1998. Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes Dev.* **12**: 3182–3194.
50. Puigserver, P., Z. Wu, C. W. Park, R. Graves, M. Wright, and B. M. Spiegelman. 1998. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell.* **92**: 829–839.
51. Wu, Z., P. Puigserver, U. Anderson, C. Zhang, G. Adelmant, V. Mootha, A. Troy, S. Cinti, B. Lowell, B. C. Scarpulla, et al. 1999. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell.* **98**: 115–124.
52. Tsuchida, A., T. Yamauchi, Y. Ito, Y. Hada, T. Maki, S. Takekawa, J. Kamon, M. Kobayashi, R. Suzuki, K. Hara, et al. 2004. Insulin/foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity. *J. Biol. Chem.* **279**: 30817–30822.
53. Chinetti, G., C. Zawadzki, J. C. Fruchart, and B. Staels. 2004. Expression of adiponectin receptors in human macrophages and regulation by agonists of the nuclear receptors PPAR α , PPAR γ , and LXR. *Biochem. Biophys. Res. Commun.* **314**: 151–158.