

Conditionally immortalized white preadipocytes: a novel adipocyte model^S

D. L. Morganstein,* M. Christian,* J. J. O. Turner,[†] M. G. Parker,* and R. White^{1,*}

Institute of Reproductive and Developmental Biology* and Metabolic Medicine,[†] Imperial College London, London W12 0NN, United Kingdom

Abstract This study describes a novel approach to generate conditionally immortalized preadipocyte cell lines from white adipose tissue (IMWAT) that can be induced to differentiate into white adipocytes even after expansion in culture. Such adipocytes express markers of white fat such as peroxisome proliferator-activated receptor γ and aP2 but not brown fat markers, have an intact insulin signaling pathway, and express proinflammatory cytokines. They can be readily transduced with adenoviral vectors, allowing them to be used to investigate the consequences of the depletion of specific adipocyte factors using short hairpin RNA. This approach has been used to study the effect of reduced expression of the nuclear receptor corepressor receptor interacting protein 140 (RIP140), a regulator of adipocyte function. The depletion of RIP140 results in changes in metabolic gene expression that resemble those in adipose tissue of the RIP140 null mouse.^S Thus, IMWAT cells provide a novel model for adipocytes that are derived from preadipocytes rather than fibroblasts and provide an alternative system to primary preadipocytes for the investigation of adipocyte function.—Morganstein, D. L., M. Christian, J. J. O. Turner, M. G. Parker, and R. White. **Conditionally immortalized white preadipocytes: a novel adipocyte model.** *J. Lipid Res.* 2008. 49: 679–685.

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The adipocyte plays a key role in the development of obesity and diabetes, functioning both as an energy store and as an endocrine tissue to regulate whole body energy balance. Key features of adipocytes include lipid storage and a rapid response to insulin stimulation that involves the activation of a number of signaling pathways, leading to diverse metabolic changes (reviewed in Refs. 1, 2). Recent evidence has also shown that adipocytes are an important source of inflammatory cytokines (3). The generation of genetically modified mice and the isolation of adipocytes from them provides an opportunity to study the signaling pathways involved in adipose biology and as

models for the pathogenesis of obesity and diabetes. Although primary cultures from adipose tissue can be readily differentiated into adipocytes (4), they have a limited lifespan and are not always suitable for molecular studies.

A number of cell lines that can be induced to differentiate into adipocytes (5) have been generated for use in such studies. The analysis of 3T3-L1 adipocytes (6) has provided many insights, but these cells are aneuploid and were derived from embryo fibroblasts rather than preadipocytes.

Procedures for the isolation of immortalized adipose cell lines from the mouse have been described (7), including the use of SV40 transforming genes (7, 8). However, SV40 T-antigen functions by deactivating proteins, including Retinoblastoma (Rb), and recent studies have shown that Rb null preadipocytes differentiate preferentially into brown adipocytes (9). It appears that members of the E2F family, themselves regulated by Rb, have roles in adipogenesis (10). Thus, this approach has limitations for the study of differentiation in general and the function of white adipocytes in particular. Indeed, there is evidence that wild-type SV40 large T-antigen can block the differentiation of preadipocytes (11). A possible solution to this problem is to use modified conditionally regulated SV40 T-antigen.

The H-2k^D-tsA58 mouse, also known as the IMMORTO™ mouse, is derived from CBA/Ca \times C57BL/10 mice genetically modified to express a mutated, temperature-sensitive SV40 large T-antigen under the control of a major histocompatibility complex class II promoter (12). A number of cell types, including myoblasts, have been derived from this mouse model (13–16); these proliferate at permissive temperature (33°C) in the presence of interferon γ but can

Abbreviations: GFP, Green Fluorescent Protein; IL-6, interleukin 6; IMBAT, immortalized brown adipocyte; IMWAT, immortalized white adipocyte; LPS, lipopolysaccharide; PRDM, PR domain containing 16; shRNA, short hairpin RNA; Rb, Retinoblastoma; TNF- α , tumor necrosis factor- α ; Ucp1, Uncoupling Protein 1.

¹To whom correspondence should be addressed.

e-mail: roger.white@imperial.ac.uk

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be induced to differentiate when cultured at higher temperature (37°C) in the absence of interferon.

In this study, we derived white preadipocytes from the H-2k^b-tsA58 transgenic mouse that proliferate in culture under permissive conditions for the T-antigen but differentiate into white adipocytes under hormonal stimulation in nonpermissive conditions. We have termed this model cell system IMWAT for immortalized white adipocytes.

METHODS

Derivation of the IMWAT cell line

The inguinal fat pad was isolated from IMMORTO™ transgenic mice and cut into 2 mm pieces, which were dispersed in 0.5 mg/ml collagenase (Sigma) and 10 mg/ml DNase (Roche) in serum-free DMEM:F12 culture medium before centrifugation at 170 *g* for 5 min to pellet the stromovascular function. The pellet was resuspended in DMEM:F12 growth medium as described below, and preadipocytes were selected by adherence to tissue culture plastic. Single-cell clones were then isolated by serial dilution. All animals were maintained according to United Kingdom Home Office regulations.

Tissue culture and adipogenic differentiation

Preadipocytes were cultured in DMEM:F12 nutrient mixture medium (Gibco) supplemented with L-glutamine, antibiotic, and antimycotic mixture (Gibco), 10% FBS (Gibco), and 20 U/ml IFN γ at 33°C in 10% CO₂. To differentiate into adipocytes, they were grown to 90% confluence and then cultured in medium without IFN γ at 37°C. At 48 h after confluence, they were induced to differentiate by treating with differentiation medium consisting of DMEM:F12 supplemented with 10% FBS, insulin (1 μ g/ml), dexamethasone (250 nM), isobutylmethylxanthine (0.5 mM), and rosiglitazone (2.5 μ M). After 48 h, the medium was replaced with fresh medium supplemented with 10% FBS and insulin and cultured for 7–10 days. Where indicated, differentiated adipocytes were treated with the β 3 agonist CL 316,243 at a concentration of 10 μ M for 5 h. 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% newborn calf serum. Differentiation was induced as described above.

Oil Red O staining

Oil Red O stain was used to confirm the presence of lipid in cells. Cells were washed with PBS, fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 15 min, and then rinsed with PBS. They were then stained with Oil Red O (in isopropanol) for 10 min and rinsed in 60% isopropanol and then PBS.

Quantitative real time PCR

Total RNA from cells was extracted with TRIZOL reagent (Invitrogen) according to the manufacturer's protocols. One microgram of the resulting RNA was treated with DNase (Sigma) and then reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Sigma) using Oligo-dT primers (Invitrogen). Quantitative real-time PCR was performed on an Opticon2 cyler (MJ Research) using JumpStart Taq Ready Mix (Sigma). Gene expression was normalized to the housekeeping gene L19, and fold differences were calculated by the $\Delta\Delta$ Ct method (17).

Western blotting

For insulin-stimulation studies, adipocytes were cultured for 4 h in serum-free DMEM supplemented with 2% fatty acid-

free BSA (Sigma) as an insulin-deprivation step. They were then treated with insulin at the indicated concentrations for 10 min.

Adipocytes were lysed in 1% SDS in PBS supplemented with Complete Protease Inhibitor (Roche) and subjected to one freeze-thaw cycle before centrifugation at 13,000 *g* for 20 min at 4°C. The supernatant, minus the top fat layer, was collected, and equal amounts of protein were electrophoresed on a 10% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes, then blocked for 1 h in TBS-Tween with 5% dried milk. Membranes were then probed with specific antibodies, and bands were visualized with ECL+ (Amersham). Antibodies against total Akt (No. 9272) and Ser473 Phospho-Akt (No. 9271S) were obtained from Cell Signaling. Tumor necrosis factor- α (TNF- α) was obtained from Calbiochem, and where indicated, adipocytes were precultured with TNF- α (10 ng/ml) for 72 h before insulin stimulation.

Undifferentiated preadipocytes were either maintained at 33°C with IFN γ or incubated at 37°C without IFN γ for 72 h before lysis as described above. Antibody against the SV40 large T-antigen was from Santa Cruz Biotechnology (sc-148).

Glucose uptake

Insulin-stimulated glucose uptake was measured as described elsewhere (18). Briefly, adipocytes were cultured in Krebs-Ringer-HEPES medium supplemented with fatty acid-free BSA and sodium pyruvate for 3 h before stimulation with the indicated concentrations of insulin for 30 min. They were then incubated with [³H]2-deoxyglucose for 5 min. Cells were then lysed with 2% SDS and quantitated by scintillation counting. Control samples were incubated with cytochalasin B (Sigma), and the mean value was subtracted from all other readings to correct for non-specific uptake. All values were expressed as fold induction over untreated controls.

Lipopolysaccharide responses

Preadipocytes and differentiated adipocytes were stimulated with chloroform-extracted *Escherichia coli* lipopolysaccharide (LPS) (Alexis). Interleukin 6 (IL-6) induction in the culture supernatant was assayed by ELISA (BD Biosciences), according to the manufacturer's instructions.

Adenoviral infection

Adenoviruses expressing either short hairpin RNA (shRNA) against receptor interacting protein 140 (RIP140) or a nontargeting random sequence were constructed with the pAdEasy system (4). Sequences of the shRNA used were siRIP (GATCCCC-AGAAGATCAAGATACCTCATTCAAGAGATGAGGTATCTTGA-TCTTCTTTTTTA) and siRandom (GATCCCCGACGTTAGCAA-TCCGAGCTCTTCAAGAGAGAGCTCGATTGCTAACGTC-TTTTTTA) (19). Adipocytes were infected with the appropriate adenovirus after 7 days of differentiation and then cultured in medium supplemented with insulin plus other ligands as indicated for a further 48 h before assaying. Equal infection was confirmed by fluorescence microscopy for the coexpressed Green Fluorescent Protein (GFP).

RESULTS

Immortalized white adipocytes express markers of white adipose tissue

IMWAT preadipocytes were maintained by continuous culture in the presence of IFN γ at 33°C for up to 25 pas-

sages. A high proportion of cells retained the ability to differentiate into adipocytes after 7 days of differentiation treatment, as assessed by light microscopy and Oil Red O staining for lipid accumulation (Fig. 1A). Notably, most differentiated cells morphologically showed a single large lipid droplet that more closely resembles *in vivo* adipocytes than the multiple small droplets observed in 3T3-L1 adipocytes (see supplementary Fig. 1). Western blotting showed that SV40 large T-antigen was present

in proliferating cells at the permissive conditions of 33°C with IFN γ but confirmed efficient degradation at the non-permissive conditions of 37°C without IFN γ before the onset of differentiation (Fig. 1B). When differentiated under nonpermissive conditions, the expression of mRNA of the adipocyte markers aP2, peroxisome proliferator-activated receptor γ 2, and the adipocytokine adiponectin increased over the 7 day period of differentiation (Fig. 1C and data not shown). In contrast, the preadipo-

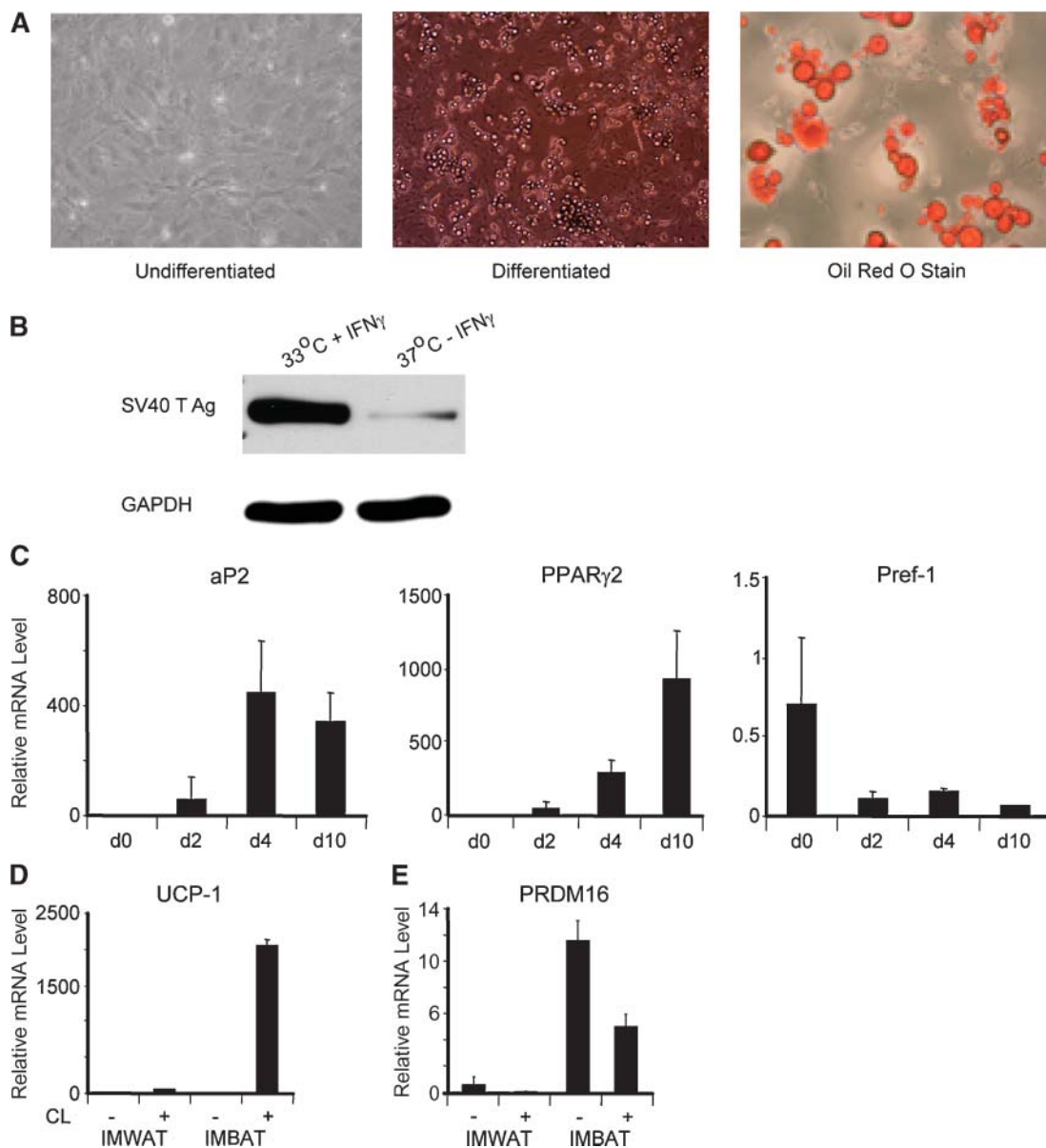


Fig. 1. Conditionally immortalized mouse white preadipocytes can differentiate *in vitro* into adipocytes. A: Photomicrographs at $\times 200$ magnification of confluent preadipocytes after withdrawal of interferon and culture at 37°C (left) and after 7 days of differentiation (middle) as described in Methods. Accumulation of lipid droplets is visible in differentiated cells, and these stain red after Oil Red O staining (right). B: Western blot showing degradation of the SV40 T-antigen in the immortalized white adipocyte (IMWAT) preadipocytes on moving from permissive to nonpermissive conditions for 48 h. C: Expression of key markers of adipocytes increases after differentiation. The y axis indicates fold change of the mRNA level compared with day 0 (relative units). PPAR, peroxisome proliferator-activated receptor. D: Uncoupling Protein (Ucp1) is expressed at very low levels in differentiated IMWATs compared with immortalized brown adipocytes (IMBATs) with and without stimulation with the β 3 agonist CL-316243. E: PR domain containing 16 (PRDM16) expression is low in the IMWAT preadipocytes and adipocytes compared with IMBATs. Error bars indicate SD.

cyte marker Pref-1 was highly expressed in the undifferentiated preadipocytes and declined with differentiation (Fig. 1C).

To confirm that differentiation had proceeded down the white lineage and that the expression of SV40 large T-antigen in proliferating cells had not influenced adipogenesis, differentiated adipocytes were examined for the absence of brown fat markers. The expression of the brown fat-specific gene Uncoupling Protein 1 (Ucp1) in IMWAT adipocytes was compared with that of an adipocyte cell line derived from brown adipose tissue of the IMMORTO™ mouse, termed IMBATs (M. Christian et al., unpublished data). As expected, IMBAT adipocytes showed a robust increase in Ucp1 expression after treatment with a β 3 agonist, CL 316,243. In contrast, Ucp1 was detected only at very low levels in the IMWAT adipocytes, even after β adrenergic stimulation (Fig. 1D). PR domain containing 16 (PRDM16) was recently described as a novel factor determining brown adipocyte differentiation that is highly expressed in brown preadipocytes (20). Importantly, the expression of PRDM16 was considerably lower in the IMWAT preadipocytes than in the conditionally immortalized brown preadipocytes (Fig. 1E).

Insulin signaling and proinflammatory cytokine production in IMWAT cells

We next investigated whether differentiated IMWAT adipocytes reproduced normal insulin signaling. Insulin-stimulated glucose uptake was assessed with [3 H]2-deoxyglucose and showed a marked stimulation by insulin in a dose-dependent manner (Fig. 2A). The induction was comparable to that obtained with 3T3-L1 adipocytes (Fig. 2A). Furthermore, Akt phosphorylation was increased by insulin stimulation, suggesting that insulin signaling was intact in the differentiated adipocytes (Fig. 2B). The proinflammatory mediator TNF- α has been shown to induce insulin resistance (21). Pretreatment of differentiated IMWAT cells with TNF- α for 3 days before insulin stimulation results in the suppression of Akt phosphorylation (see supplementary Fig. II).

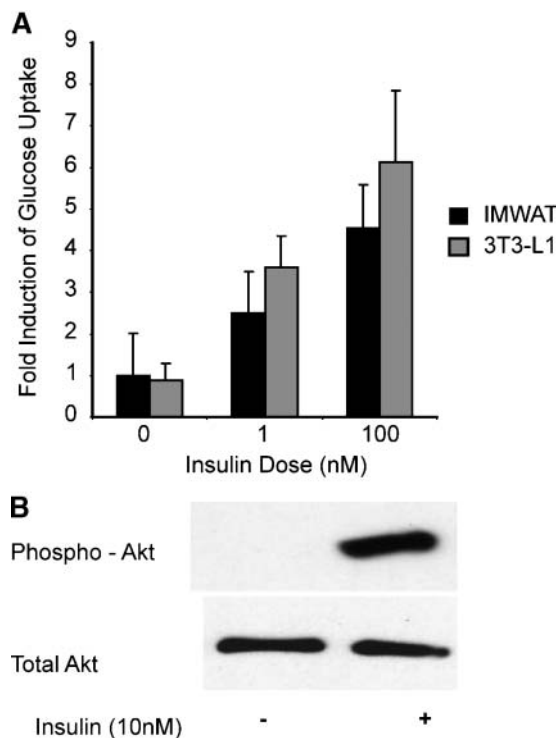


Fig. 2. Insulin signaling is intact in differentiated IMWATs. A: Differentiated adipocytes were serum-starved overnight and then stimulated with insulin at the indicated doses for 30 min before assessing the uptake of [3 H]2-deoxyglucose. Data are presented as mean induction over untreated controls. Error bars indicate SD. Uptake into control adipocytes treated with cytochalasin B was subtracted from all readings to control for nonspecific uptake. B: Western blot showing phosphorylation of Akt in untreated adipocytes and after 10 min of stimulation with insulin. The bottom panel shows total Akt protein in the same samples.

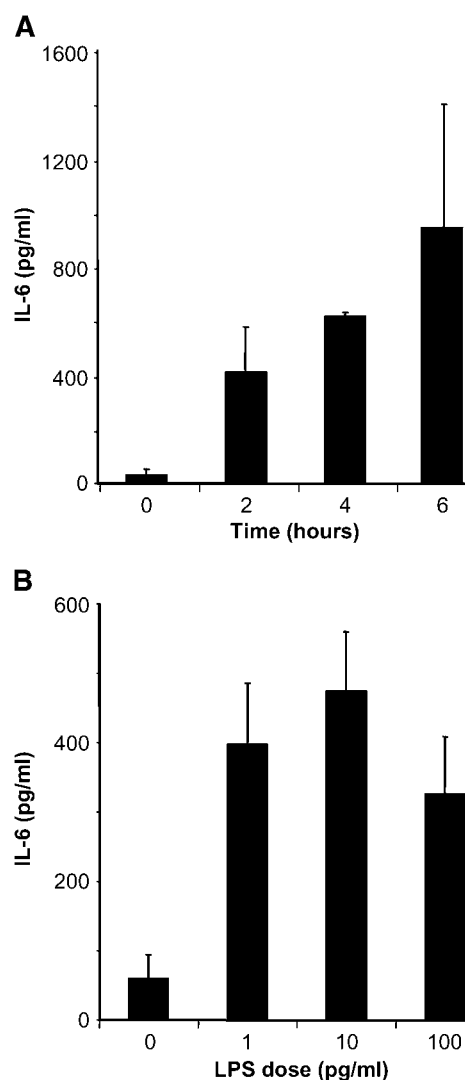


Fig. 3. Interleukin 6 (IL-6) production by differentiated adipocytes. A: Differentiated adipocytes were treated with 10 ng/ml lipopolysaccharide (LPS), and supernatants were collected at the basal time point and after 2, 4, and 6 h for IL-6 assay. B: Adipocytes were treated with varying doses of LPS, and supernatants were collected after 6 h for IL-6 assay. Error bars indicate SD.

It is now recognized that adipocytes are also a source of inflammatory cytokines (22). Therefore, we investigated the inflammatory response of differentiated IMWAT adipocytes to LPS stimulation. IL-6 production was increased in a dose-dependent manner, and levels increased progressively after LPS stimulation (Fig. 3).

Depletion of endogenous RIP140 expression in IMWAT cells

IMWAT adipocytes were then used to study the effects of depleting RIP140 from a fully differentiated fat cell. Adipocytes were transduced with recombinant adenoviruses expressing both the GFP marker to monitor infection efficiency and shRNA against either the nuclear receptor corepressor RIP140 or a random, nontargeting sequence. Fluorescence microscopy demonstrated a high efficiency of infection of differentiated adipocytes and the fact that mostly differentiated cells were infected, as

detected by GFP expression. IMWAT adipocytes infected efficiently at a multiplicity of infection of 100, at least as well as 3T3-L1 adipocytes (Fig. 4A; see supplementary Fig. III). Western blot for RIP140 protein levels confirmed the ability of the adenovirally expressed shRNA to deplete the cells of RIP140 (Fig. 4B).

The depletion of RIP140 in IMWAT-derived adipocytes resulted in a marked increase in both basal and $\beta 3$ adrenergic stimulated Ucp1 expression, together with the upregulation of other RIP140 target genes such as CPT1b, CIDEA, and COX8b (Fig. 4C).

DISCUSSION

We have shown that conditionally immortalized white preadipocytes derived from the H-2k^b-tsA58 transgenic

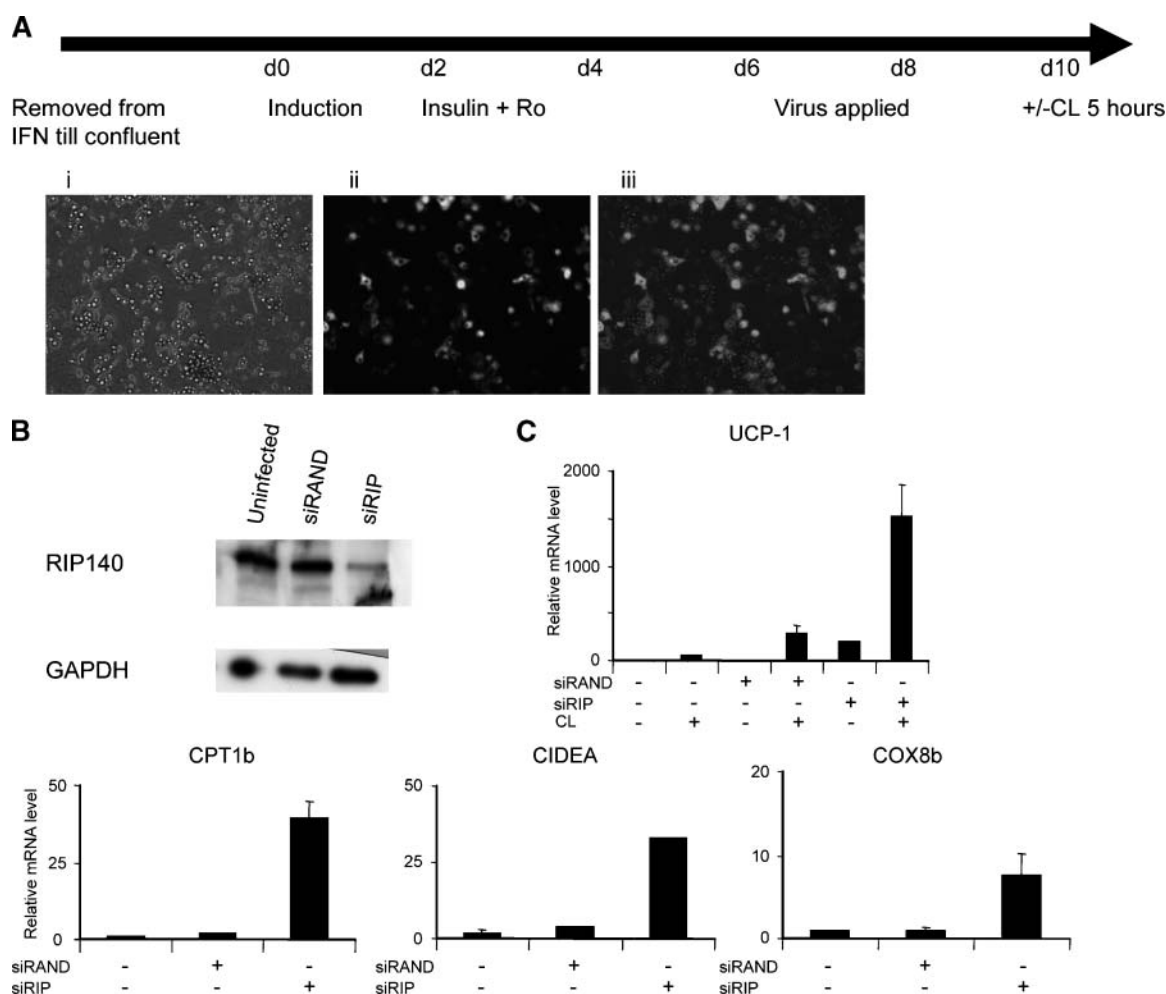


Fig. 4. Depletion of receptor interacting protein 140 (RIP140) in differentiated IMWAT adipocytes results in the upregulation of known RIP140 target genes, including Ucp1. **A:** Timeline of the experiment showing the differentiation protocol and the timing of adenovirus infection. Ro, rosiglitazone; CL, $\beta 3$ agonist CL-316243. The photomicrographs below show differentiated adipocytes (i) and Green Fluorescent Protein expression in infected adipocytes (ii); the merged image (iii) confirms that infection is limited to differentiated cells and that a high proportion of differentiated adipocytes are infected. **B:** Western blot confirming the knockdown of RIP140 by siRIP adenovirus but not by siRandom (siRAND). **C:** Expression analysis in a clone IMWAT D2 after infection with adenovirus expressing either siRIP or siRandom and treatment with rosiglitazone. Expression of Ucp1, CIDEA, CPT1b, and COX8b mRNA increases after the depletion of RIP140. The y axis indicates fold change (relative units). Results are averages of four experiments and were confirmed in a second clone. Error bars indicate SD.

mouse retain the ability to differentiate into white adipocytes. The lack of Ucp1 expression in the differentiated cells, together with the low levels of expression of PRDM16, compared with a brown preadipocyte line, demonstrate that the presence of the heat-labile T-antigen before differentiation does not disrupt the process of white adipocyte differentiation, suggesting that these conditionally immortalized preadipocytes are a valid model in which to study white adipocytes. Furthermore, the preadipocyte phenotype was stable even after prolonged culturing, with a substantial proportion of the cells retaining the ability to differentiate after 25 passages, compared with the very limited lifespan of primary preadipocytes.

The differentiated adipocytes also retained insulin-stimulated glucose uptake and exhibited an inflammatory response to the Toll-Like Receptor-4 ligand LPS, suggesting that they replicate many aspects of normal adipocyte physiology.

The approach described in this study has the potential to be applied to the analysis of the role of key regulatory factors in white adipose cells by using either transgenic or null mouse models crossed with the H-2k^b-tsA58 strain. However, it is also important to be able to manipulate the expression of key factors only after differentiation has been completed, for example, by using small interfering RNA to deplete wild-type cells of specific factors. A major disadvantage of many adipocyte models is the difficulty in achieving efficient transfection. Adenoviruses are reported to infect a wide variety of cell types and have been reported to infect differentiated adipocytes, although a high multiplicity of infection is required (23). We have found that differentiated IMWAT adipocytes are readily infected by recombinant adenoviruses at a multiplicity of infection of 50–100. IMWAT adipocytes were transduced by recombinant adenoviruses, and shRNA was used to modify gene expression.

Using this approach, we have determined the effect on gene expression of knockdown of the nuclear receptor corepressor RIP140. Lack of RIP140 in vivo (24), and in cell lines derived from RIP140 null mice (25), results in the upregulation of numerous genes involved in energy dissipation in white fat, including the expression of Ucp1. Depletion of RIP140 from 3T3-L1 adipocytes results in the upregulation of many of the same genes increased in the RIP140 null mouse, with the exception of Ucp1 (18). Thus, a major concern with some existing models of adipocytes is that they may not truly reflect patterns of gene regulation found in adipose tissue. Preliminary studies with the IMWAT cells demonstrate that Ucp1 expression was increased in the absence of RIP140 (4). In this report, we demonstrate that in IMWAT adipocytes, the response to the depletion of RIP140 closely resembles the changes seen in primary adipocytes from RIP140 null mice and in explants of white fat depleted of RIP140 (26). This includes an upregulation in the expression of CIDEA, the mitochondrial protein COX8b, and Ucp1 and suggests that IMWAT adipocytes replicate the in vivo situation more accurately than 3T3-L1 adipocytes, at least in terms of the transcriptional control of metabolic genes.

In summary, we have used an in vivo approach to derive preadipocyte cells that can be maintained in cell culture. Such conditionally immortalized white preadipocytes provide a novel in vitro model of white adipocytes with advantages over more conventional fibroblast-derived cell lines but without many of the limitations of primary preadipocyte cultures. This cell system should facilitate further studies of adipogenesis and enable investigation of the roles of important factors essential for normal adipocyte function.

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