Regulation of human subcutaneous adipocyte differentiation by EID1

Diana Vargas¹, Noriaki Shimokawa², Ryosuke Kaneko³, Wendy Rosales¹, Adriana Parra¹, Ángela Castellanos¹, Noriyuki Koibuchi² and Fernando Lizcano¹

¹Center of Biomedical Research (CIBUS), Universidad de La Sabana, Km. 7 Autopista Norte de Bogotá, 140013 Chia, Colombia

²Department of Integrative Physiology, Gunma University, Maebashi, Japan

³Institute of Experimental Animal Research, Gunma University, Maebashi, Japan

Correspondence should be addressed to F Lizcano

Email
fernando.lizcano@unisabana.edu.co

Abstract

Increasing thermogenesis in white adipose tissues can be used to treat individuals at high risk for obesity and cardiovascular disease. The objective of this study was to determine the function of EP300-interacting inhibitor of differentiation (EID1), an inhibitor of muscle differentiation, in the induction of beige adipocytes from adipose mesenchymal stem cells (ADMSCs). Subcutaneous adipose tissue was obtained from healthy women undergoing abdominoplasty. ADMSCs were isolated *in vitro*, grown, and transfected with EID1 or EID1 siRNA, and differentiation was induced after 48 h by administering rosiglitazone. The effects of EID1 expression under the control of the aP2 promoter (aP2-EID1) were also evaluated in mature adipocytes that were differentiated from ADMSCs. Transfection of EID1 into ADMSCs reduced triglyceride accumulation while increasing levels of thermogenic proteins, such as PGC1 α , TFAM, and mitochondrial uncoupling protein 1 (UCP1), all of which are markers of energy expenditure and mitochondrial activity. Furthermore, increased expression of the beige phenotype markers CITED1 and CD137 was observed. Transfection of aP2-EID1 transfection induced the conversion of mature white adipocytes to beige adipocytes, as evidenced by increased expression of PGC1 α , UCP1, TFAM, and CITED1. These results indicate that EID1 can modulate ADMSCs, inducing a brown/beige lineage. EID1 may also activate beiging in white adipocytes obtained from subcutaneous human adipose tissue.

Key Words

- ▶ retinoblastoma
- ▶ ADMSCs
- ▶ PPAR γ
- ▶ EID1
- ▶ beige fat

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Introduction

Obesity and its associated metabolic diseases, such as type 2 diabetes mellitus, hypertension, and cardiovascular disease, are increasing in prevalence due to a combination of environmental factors and genetic variations (van Dijk *et al.* 2014, Robbins *et al.* 2014). Recent research has led to a deeper understanding of adipocyte physiology. These advances may provide important clues to find effective options for treating obesity (Nedergaard *et al.* 2011, Wu *et al.* 2013). Adipocytes develop from adipose mesenchymal stem cells (ADMSCs), which are primarily of mesodermal origin. However, the specific localizations

of fat cells and their associated differential gene expression patterns, thermogenic capacities, and abilities to induce vascular complications suggest that they have diverse origins. Indeed, various types of adipocytes were recently identified in adults (Sidossis & Kajimura 2015). In contrast with white adipose tissue (WAT), brown adipose tissue (BAT) is specialized for the dissipation of chemical energy in the form of heat, a process dependent on mitochondrial uncoupling protein 1 (UCP1). Adult humans lack classical BAT but possess a subset of cold-inducible beige adipocytes in their WAT. The recognition of the phenotypic plasticity

of beige fat cells in adults has increased interest in the development of potential drugs to treat obesity and metabolic diseases (Cannon & Nedergaard 2004, Rosen & Spiegelman 2014). The origin of beige fat is unclear, but the progenitors of several types of these cells are similar to progenitors of white adipocytes (Seale *et al.* 2008, Wu *et al.* 2012). Thus, subsets of the predominant white fat cells in adults may be converted to the more metabolically active beige fat cells (Sharp *et al.* 2012, Lidell *et al.* 2013, Sidossis & Kajimura 2015). Although the molecular mechanisms underlying this process remain unclear, producing metabolically active, physiological beige cells from adult white cells represents an interesting challenge (Cypess *et al.* 2009, Vitali *et al.* 2012, Berry & Rodeheffer 2013, Lidell *et al.* 2014).

The differentiation of beige adipocytes from precursor cells and WAT beiging transdifferentiation from white adipocytes is regulated by many factors, including the bone morphogenetic protein 7/PR domain containing protein 16/peroxisome proliferator-activated receptor gamma (PPAR γ) coactivator 1 alpha (BMP7/PRDM16/PGC1 α) axis (Seale *et al.* 2008, Seale 2015). Moreover, agonists of nuclear receptor PPAR γ and inhibitors of retinoblastoma protein (pRb) play important roles in the development and activation of beige cells (Auffret *et al.* 2012, Ohno *et al.* 2012, Harms & Seale 2013). The inactivation of pRb may increase levels of PGC1 α (Hallenborg *et al.* 2009, Mercader *et al.* 2009, Lee *et al.* 2013). Studies in animal models show that the inactivation of pRb in adipose tissue increases mitochondrial activity levels and induces resistance to obesity (Hansen *et al.* 2004a,b, Khidr & Chen 2006, Dali-Youcef *et al.* 2007, Calo *et al.* 2010, Auffret *et al.* 2012).

In searching for molecules that modulate adipogenesis, we identified EP300-interacting inhibitor of differentiation (EID1; Lizcano & Vargas 2010), which was originally described as an inhibitor of muscle cell differentiation (MacLellan *et al.* 2000, Miyake *et al.* 2000). EID1 reduces the transactivation of several nuclear receptors, including glucocorticoid receptor, thyroid receptor, and estrogen receptor (Miyake *et al.* 2000, Bavner *et al.* 2002). However, little is known regarding the role of EID1 in the regulation of PPAR γ . EID1 is thought to mediate small heterodimer partner (SHP) repression (Tabbi-Annani *et al.* 2010), with SHP being a predominant activator of PPAR γ (Nishizawa *et al.* 2002). EID1 contains an LxCxE sequence in its C-terminal domain, allowing it to bind to pRb (Wen & Ao 2001), and an acidic site that inhibits p300-CBP coregulators. By inactivating pRb and reducing PPAR γ activity, EID1 activates the expression of

thermogenic proteins in 3T3-L1 cells (Lizcano & Vargas 2010). These findings suggested that EID1 may play an important role in the differentiation of brown and beige adipocytes.

This study was designed to assess the effects of EID1 on both mesenchymal cells and mature adipocytes induced from subcutaneous fat of a group of healthy women. EID1 overexpression promoted the activation of beige adipocytes, indicating that EID1 may be a critical factor in regulating brown/beige adipocyte differentiation. Moreover, EID1 may be a therapeutic target for obesity by stimulating the conversion of white to beige adipocytes, thus increasing energy expenditure.

Subjects and methods

Subjects

Subcutaneous fat samples were obtained from ten healthy women between 20 and 40 years of age who underwent abdominoplasty. Each woman had a BMI between 23 and 25 kg/m² and had not been treated with any drugs for at least 3 months prior to sampling. Their lipid profiles and blood glucose levels were normal. All subjects received detailed information regarding the purpose of the study and provided written informed consent. The study protocol was approved by the Ethics Committee of La Sabana University.

Plasmids

PPAR γ was cloned into the Sall site of the pSV.SPORT1 vector. The PPARE-Luc and Gal4-PPAR γ -LBD constructs were previously described (Spiegelman 1998). EID1 was cloned into the expression vector pcDNA3.1-T7 at the BamHI and EcoRI restriction sites. EID1-157 was generated by PCR using the primers 5'-GTGAATTCCACAATGTCGGAAATGGCTG-3' (forward) and 5'-AGCAATCTGATCAAACGGGGTCTTCTC-3' (reverse) and inserting the PCR product into the vector TOPO pcDNA 3.1 (Life Technologies).

The Δ EID1 mutant lacks both the C-terminal domain (LxCxE) that allows the protein to bind to pRb and the acidic residues 53–63 and 92–115 that facilitate binding to p300 (Miyake *et al.* 2000). The p300 plasmid was constructed by inserting the sequence encoding p300 into the NotI and HindIII sites in the NHA-CMV vector. Construction of the SRC1 and PRIP/nuclear receptor coactivator 6 (NCOA6) plasmids was previously described (Onate *et al.* 1995, Zhu *et al.* 2000). The 5 $\times$ UAS reporter

was cloned into the BamHI and HindIII restriction sites in the PT109 vector, which contained the thymidine kinase promoter coupled to the luciferase gene (Takeshita *et al.* 1997). To construct the aP2-EID1-woodchuck hepatitis virus post-transcriptional regulatory element (WPRES) plasmid, a 5.4 kb region identified as the aP2 promoter was amplified by PCR from mouse genomic DNA using Tks Gflex DNA Polymerase (Takara, Otsu, Shiga, Japan), according to the manufacturer's instructions, and specific primers (Imai *et al.* 2001) also containing 5' SalI and 3' NheI restriction sites, and the product was cloned into pCR-Blunt II TOPO (Invitrogen). The previously described WPRES sequence (Zufferey *et al.* 1999) was digested with EcoRI and inserted into pCX-EGFP (Clontech), generating pCX-WPRES. EID1 was obtained by PCR using the primers 5'-ACAATGTCGGAATGGCTG-3' (forward) and 5'-GAGATTATTGATAGAGAGTAG-3' (reverse), containing NheI and SacI ends respectively, and the product was inserted into pCX-WPRES, resulting in pCX-EID1-WPRES. The aP2 promoter was cloned upstream of pCX-EID1 between the 5' SalI and 3' NheI sites, generating the 7.2 kb pCX-aP2-EID1-WPRES plasmid.

Cell culture

U2OS cells obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies) and 1% penicillin/streptomycin at 37 °C in an atmosphere containing 5% CO₂. Primary cultures were prepared from 20 g abdominal subcutaneous adipose tissue. Fat samples were washed with PBS, and fibrous material and all visible blood vessels were removed. The samples were incubated with 250 U/ml collagenase type I, 20 mg/ml BSA, and 60 µg/ml gentamycin in PBS for 90 min at 37 °C while stirring. The samples were centrifuged at 200 *g* for 10 min, and the pellet was suspended in lysis buffer, consisting of erythrocytes, 154 mM ammonium chloride (NH₄Cl), 5.7 mM monobasic potassium phosphate (K₂HPO₄), and 0.1 mM EDTA, pH 7.3, for 10 min. This mixture was filtered through a 150 µm pore size nylon mesh, followed by centrifugation at 200 *g* for 10 min. The cell pellet was suspended at 10 000 cells/cm² in growth medium, consisting of DMEM/F12 plus 10% FBS and 50 mg/ml gentamicin. After 24 h, the cells were washed and resuspended in PM4 proliferation medium (DMEM/F12, 2.5% FBS, 1 ng/ml basic fibroblast growth factor, 10 ng/ml epidermal growth factor, and 8.7 µM insulin; Skurk *et al.* 2007) until they became 100% confluent and had differentiated into mature adipocytes.

These cells were identified by incubation with antibody to CD34 and sorting by flow cytometry (Rodeheffer *et al.* 2008). Human mesenchymal cells were differentiated into adipocytes by incubation for 72 h in DMEM/F12 containing 66 nM insulin, 1 nM triiodo-L-thyronine, 10 µg/ml transferrin, 0.5 mM isobutylmethylxanthine, 1 µM dexamethasone, and 2 µM rosiglitazone. The medium was replaced with basal medium containing equal concentrations of insulin, triiodo-L-thyronine, and transferrin, and the cultures were grown for an additional 10 days, with the medium being replaced every 3 days (Skurk *et al.* 2007).

Transfections

Functional assays for luciferase activity were performed using the U2OS cell line. The cells were transfected at 80% confluence using Lipofectamine 2000 (Life Technologies) with 0.85 µg PPRE-Luc, 0.5 µg CMV-b-galactosidase, 0.1 µg PPARγ, 0.2 µg EID1, and 0.1 µg of each of the coactivators. After 24 h, the cells were treated with 1 µM rosiglitazone or DMSO as vehicle. After an additional 24 h, the cells were lysed, and both luciferase activity and β-galactosidase levels were measured. The results were reported as fold changes in luciferase gene activity compared with the empty expression vector, which was used as a control.

Transfections of the ADMSCs with the EID1 expression vector and with empty vector (pcDNA 3.1) were performed using Lipofectamine LTX (Life Technologies), according to the manufacturer's instructions. Briefly, 24 h before transfection, 0.2 × 10⁶ cells were subcultured in each well of a 24-well culture plate containing growth medium that was free of antibiotics. The transfection mixture was prepared in 50 ml Opti-MEM medium (Life Technologies) with 0.5 µg DNA and 0.5 µl Plus Reagent; Lipofectamine was added to 50 ml Opti-MEM medium at a 1:4 DNA:Lipofectamine ratio. After 20 min, the mixture was added to the cells. The medium was removed after 4 h and replaced with complete growth medium. After 48 h, differentiation was induced for 10 days, and triglyceride (TG) analysis was performed as previously described (Lizcano & Vargas 2010). For protein expression analysis, total protein was isolated 48 h after transfection, which corresponded to day 0. Differentiation was induced, and protein extracts were obtained after 3 and 6 days.

To determine the effects of EID1 on mature adipocytes, ADMSCs were induced to differentiate into adipocytes. pCX-aP2-EID1-WPRES and pCX-WPRES as a

control were overexpressed by Lipofectamine LTX using a reverse transfection method as described (Kilroy *et al.* 2009). The transfection mixture was prepared in 100 μ l Opti-MEM with 2.5 μ g DNA and 2.5 μ l Plus Reagent. Lipofectamine was diluted in 100 μ l Opti-MEM at a 1:5 ratio of DNA:Lipofectamine. After 20 min, 200 μ l solution was added to 1.5×10^6 fat cells suspended in each well of a six-well plate. After 4 h, the medium was replaced by complete differentiation medium containing 1 μ M rosiglitazone. The cells were maintained in growth medium for 24 or 48 h.

Flow cytometry

ADMSCs (5×10^5) transfected with the indicated plasmids were harvested and incubated with a 1:100 dilution of anti-CD137-PE (4B4-1; BD Biosciences, Piscataway, NJ, USA) or control isotype IgG1 MABs for 20 min in the dark. After several washes with PBS, the samples assessed by flow cytometry (fluorescence-activated cell sorting; Roche), with the results analyzed by FlowJo Software (Treestar, Ashland, OR, USA).

QRT-PCR

Total RNA from cultured human ADMSCs was isolated 24 h after EID1 transfection and 3 and 6 days after induction of differentiation. RNA was extracted from cells using the High Pure RNA Isolation Kit (Roche Diagnostics), according to the manufacturer's instructions. First-strand cDNA was synthesized from 500 ng of total RNA using Transcriptor First Strand cDNA Synthesis Kits (Roche Diagnostics). PCRs were performed using primers specific for human PGC1 α (forward, 5'-CTGTGTCACCAACCCAAATCCTT-AT-3'; reverse, 5'-TGTGTCGAGAAAAGGACCTTGA-3'), human UCP1 (forward, 5'-GTGTGCCCAACTGTGCAATG-3'; reverse, 5'-CCAGGATCCAAGTCGCAAGA-3'), human CITED1 (forward, 5'-CAACCTTGCGGTGAAAGATCG-3'; reverse, 5'-GGAGAGCCTATTGGAGATCCC-3'), and human GAPDH (forward, 5'-ACCCACTCCTCCACCTTTGAC-3'; reverse, 5'-TGTTGCTGTAGCCAAATTCGTT-3') and FastStart Essential DNA Green Master (Roche Diagnostics). The levels of expression of PGC1 α , UCP1, and CITED1 were normalized relative to the levels of expression of GAPDH in the same samples.

siRNA

Synthetic siRNA oligonucleotides were designed and produced by Invitrogen. After testing three sequences to determine the most effective sequence for blocking EID1

expression, the sequence used was 5'-GAGAGACAGUGAGAUCCUGCAGGAU-3', identified as siRNAEID1/2. As a control, cells were treated with Lipofectamine alone or siRNAEID1/1, which did not affect EID1 expression. Primary cells were transfected at 90% confluence with 30 nM siRNA using 1.2 μ l Lipofectamine RNAi MAX, according to the manufacturer's instructions. After 48 h, total protein was isolated, and EID1 and PGC1 α levels were evaluated.

Western blot analysis

Total protein from cultured human ADMSCs was extracted 48 h after EID1 transfection and 3 and 6 days later using RIPA buffer (Abcam, Cambridge, MA, USA) and 1 mg protease inhibitors (Roche Diagnostics). Protein concentrations were quantitated using the Bradford method, and 50 mg aliquots of proteins were denatured at 95 $^{\circ}$ C and subjected to PAGE. The proteins were electrophoretically transferred to PVDF membranes that had been pre-treated with 100% methanol for 2 min. Membrane blocking was performed with PBS-T (1 $\times$ PBS and 0.1% Tween 20) containing 5% powdered skim milk. The membranes were subsequently incubated with the following antibodies: rabbit anti-PGC1 α , 1:1000 dilution (ab54481, Abcam); rabbit anti-UCP1, 1:1000 dilution (ab1551117, Abcam); rabbit anti-TFAM (7495), 1:1000 dilution (Cell Signaling, Beverly, MA, USA); mouse anti-CITED1 (ab87978, Abcam), 1:250 dilution; and mouse anti-pRb (MAB3168, Millipore, Concord, MA, USA), 1:2000 dilution. The membranes were washed and incubated with HRP-conjugated secondary antibodies against rabbit IgG, at dilutions of 1:2000 and 1:3000 to detect expression of PGC1 α , TFAM, and UCP1, or with HRP-conjugated anti-mouse IgG, at a dilution of 1:2000, to detect expression of CITED and pRb. Antibody binding was detected by chemiluminescence, using Crescendo Luminata Kits (EDMillipore, Darmstadt, Germany), according to the manufacturer's instructions. Images were captured and analyzed using a myECL Imager (Thermo Scientific, Rockford, IL, USA). The results were expressed as mean $\pm$ S.E.M. and analyzed by ANOVA, with differences considered statistically significant when the *P* value was <0.05 .

Differences in ADMSCs transfected with pCX-aP2-EID1-WPRE and pCX-WPRE were compared. Mature cells were lysed with RIPA buffer 24 and 48 h after transfection, and total protein extracts obtained. These extracts were electrophoresed on SDS-PAGE and electrophoretically transferred to PVDF membranes as described above.

Figure 1

EID1 expression in fat cells and effects of EID1 on PPARγ transactivation and coregulators. (A) Samples of intercapular, inguinal, and visceral fat were obtained from 2-month-old female Wistar rats weighing 150 g. (B) Samples from normal women were obtained from their subcutaneous fat and ADMSCs were identified and induced to differentiate into fat cells. Protein extracts were obtained before and after 10 days of induced differentiation. (C) The Gal4-PPARγ-LBD expression vector (0.1 μg) was cotransfected with the 5×UAS reporter (0.85 μg) and EID1 expression vector (0.2 μg) in the presence or absence of rosiglitazone (1 μM). After 24 h, luciferase activity

was measured. (D) U2OS cells were cotransfected with the expression plasmid encoding PPARγ (0.1 μg) in the presence of each of the coactivators (0.1 μg), the EID1 vector (0.2 μg), and the PPRE-Luc reporter (0.1 μg). These cells were treated with 1 μM rosiglitazone for 24 h, after which luciferase activity was measured. Luciferase activity levels were normalized relative to that of CMV-b-galactosidase (0.5 μg). These results are representative of three independent experiments with triplicate samples. * $P < 0.05$.

The expression of FABP4 was assayed using rabbit anti-FABP4 antibody (ab92501, Abcam), diluted 1:1500, and HRP-conjugated rabbit IgG secondary antibody diluted 1:3000.

Statistical analysis

Cells obtained from the ten healthy women were assessed individually ($n = 10$), with data expressed as the mean $\pm$ s.d. Results were compared by ANOVA, followed by the Bonferroni's *post-hoc* test. All statistical analyses were performed using SPSS Software, with a P value < 0.05 considered statistically significant.

Results

Expression of EID1 in adipose tissue and effect of EID1 on PPARγ activation

To investigate the expression of EID1 in different types of fat, sample from intercapular, inguinal, and visceral regions was obtained from Wistar rats and total proteins were isolated. The highest levels of EID1 protein were detected in brown fat from intercapular adipose tissue, whereas inguinal and visceral fat showed low levels of EID1 (Fig. 1A). In addition, western blotting showed that EID1 protein was expressed by both precursors and adipocytes induced from subcutaneous fat (Fig. 1B).

Figure 2

Overexpression of EID1 reduces lipid accumulation in ADMSCs. (A) Cells obtained from human subcutaneous adipose tissue were transfected with 0.5 μg of empty vector (pCDNA 3.1), EID1 (0.5 μg), EID1-157, or ΔEID1, 0.5 μl of Plus Reagent and Lipofectamine LTX at a ratio of 1:4. After 48 h, differentiation was induced. Ten days later, the cells were stained with Oil Red O, and triglyceride concentrations were quantitated. The negative control represent cells not induced to undergo adipogenesis.

Positive controls included cells transfected with empty vector and induced to differentiate. * $P < 0.05$ of mean $\pm$ s.d. of three independent experiments conducted in triplicate. (B) Relative levels of triglycerides in ADMSC, after inducing differentiation of fat cells and transfection with different plasmids of EID1 and mutants. To quantify triglyceride, 1 ml of isopropanol was added for 5 min, to distaining the fat deposits. Absorbance was measured at 510 nm wavelength.

Treatment with rosiglitazone of U2OS cells cotransfected with plasmids containing Gal4-PPARG-LBD and the reporter gene EID1-5×UAS-Luc showed that EID1 reduced PPARG activity by more than 50% (Fig. 1C). To assess the effects of EID1 on PPARG coactivators, U2OS cells were cotransfected with the SRC1, p300 or PRIP expression vector, the PPARG plasmid, and the PPRE-Luc promoter vector. The increased PPARG activity induced by different coactivators was reduced by EID1; however, PPARG activity was significantly reduced in the presence of p300 and PRIP, but not in the presence of SRC1 (Fig. 1D).

Overexpression of EID1 modulates adipogenic differentiation in ADMSCs

EID1 reduces TG accumulation in 3T3-L1 mouse pre-adipocytes (Lizcano & Vargas 2010). To further determine the physiological role of EID1 in adipocyte differentiation, EID1 was evaluated in ADMSCs isolated

from human adipose tissue. ADMSCs, identified by CD34 flow cytometry (data not shown), were transfected with EID1 and control plasmids, with differentiation initiated 48 h later. Ten days later, staining and TG quantification were performed.

Consistent with reports evaluating 3T3-L1 pre-adipocytes, EID1 significantly reduced TG levels in cells isolated from human adipose tissue. ΔEID1 and EID1-157 mutants, which lack the capacity to bind to pRb and p300 respectively, had no effect on adipose cell differentiation (Fig. 2A and B). As EID1 was shown to reduce the activity of pRb, the effect of EID1 on pRb in ADMSCs was evaluated. Total protein was isolated from these cells 48 h after transfection, total protein was extracted, and pRb expression was measured (Fig. 3A). Knockdown of EID1 with EID1 siRNA reduced the expression of PGC1α (Fig. 3B).

To assess the effects influence of EID1 on thermogenic proteins, cells were stimulated to differentiate 48 h after transfection. Samples were collected the day induction of

Figure 3

EID1 reduces the expression of pRb and increases thermogenic proteins in ADMSCs. (A) EID1 expression vector was transfected into ADMSCs. After 48 h, total protein was isolated, and pRb was measured by western blot. Quantitative analyses were performed by the densitometry of the bands representing the different experiments. The results were compared to the control pre-adipocytes treated only with Lipofectamine LTX or to empty pcDNA3.1 vector. (B) Endogenous EID1 expression was inhibited by transfecting the ADMSCs with siRNA against EID1 using Lipofectamine RNAiMAX. EID1 inhibition was evaluated 48 h after transfection by western blotting to detect PGC1α and EID1 levels. Control cells corresponded to cells treated with only Lipofectamine RNAiMAX and siRNAEID1/1, which had no effects on the inhibition of EID1. The reduction of EID1 was accompanied

by the decreased expression of PGC1α as shown by siRNA1/2. (C) ADMSCs were cotransfected as indicated in Fig. 2, and total protein was recovered at day 0 (48 h post-transfection), at which point differentiation was induced. The protein extracts were obtained on the indicated days. Using western blotting, the expression levels of the PGC1α, UCP1, CITED1, and TFAM proteins were measured. EID1 (–) corresponds to cells transfected with the empty pcDNA-3.1 vector. (D) The relative intensity levels were determined by the densitometry of the bands. The data are expressed as the mean ± s.d. (n = 10), and *P < 0.05 that correspond to a significant difference in relation to each day and the effects of EID1 on the proteins detected by the western blot analysis (PGC1α, UCP1, and TFAM CITED1). The data were normalized to GAPDH.

Figure 4

EID1 increases the expression of genes involved in thermogenesis. EID1 was transfected into ADMSCs, as indicated in Fig. 2. At 24 h after transfection, differentiation was induced and total RNA was isolated. The levels of expression of (A) PGC1 α , (B) UCP1, and (C) CITED were measured.

EID1 (-) corresponds to cells transfected with empty vector pcDNA 3.1. Data are expressed as mean $\pm$ s.d. and the asterisk indicates statistically significant differences ($P < 0.05$) in relation to each day and the presence or absence of EID1. GAPDH was used as a reference.

differentiation was started, with additional samples obtained 3 and 6 days later. EID1 significantly upregulated UCP1, mitochondrial PGC1 α and TFAM marker expression levels compared with cells treated with empty vector (Fig. 3C). In addition, the expression of a specific marker of beige cells, CITED1 (Fig. 3C), was increased, and surface expression of CD137 (Fig. 3E) was observed (Wu *et al.* 2012). These proteins are characteristic of the beige adipocyte phenotype and are not expressed in brown adipocytes. Quantitative RT-PCR assays for the expression of specific RNAs showed that the levels of

expression of PGC1 α , UCP1, and CITED1 were increased (Fig. 4A and B).

EID1 induces the beige phenotype in mature fat cells

To evaluate the effects of EID1 on mature white adipose cells differentiated from ADMSCs, these cells, expressing FABP4, were transfected with the pCX-aP2-EID1-WPRE transgene or the pCX-WPRE control. Proteins were extracted after 24 and 48 h, and the levels of expression levels of proteins and thermogenic beige phenotype

Figure 5

EID1 induces the beige phenotype in mature fat cells. (A) Diagram of the pCX aP2-EID1-WPRE transgene. The aP2 promoter (0.56 kb) was located upstream of the EID1 cDNA, and the WPRE gene sequence (0.54 kb) was downstream of aP2-EID1. (B) ADMSCs induced to undergo adipogenesis were transfected with the pCX-WPRE vector (2.5 μ g) or aP2-EID1-WPRE vector and Lipofectamine LTX at 1:5 ratio. At 24 or 48 h after transfection,

total proteins were isolated to detect the expression levels of PGC1 α , UCP1, TFAM, FABP4, and CITED1, as determined by western blot analysis. (C) Relative intensity levels, as determined by the densitometry of the bands. Data are expressed as the mean $\pm$ s.d. ($n = 10$). * $P < 0.05$ significant differences between pCX-WPRE and pCX-aP2 EID1-WPRE at 24 and 48 h post-transfection.

markers were measured. Treatment of differentiated cells with EID1 increased the levels of PGC1 α , UCP1, and TFAM mRNAs and significantly increased the level of expression of CITED1, a marker of beige adipose cells (Fig. 5B and C).

Discussion

The results of this study showed that EID1 modulates the differentiation of adipocyte precursors, inducing those of the brown/beige lineage. In addition, EID1 can induce the conversion of white to beige adipocytes. Increased levels of EID1 in ADMSCs increased the expression of mitochondrial proteins and regulators of thermogenesis, such as UCP1, TFAM, and PGC1 α . EID1 was initially described as an inhibitor of the differentiation of muscle cells (MacLellan *et al.* 2000, Miyake *et al.* 2000), and we recently showed that EID1 reduces the differentiation of adipocytes from 3T3-L1 pre-adipocytes (Lizcano & Vargas 2010). Treatment of ADMSCs obtained from a group of healthy women with EID1 induced adipose cell differentiation toward more metabolically active fat cells, with increased levels of expression of UCP1 and CITED1, which are specific markers of beige cells (Sharp *et al.* 2012). EID1 partially reduced the activity of PPAR γ by inhibiting the p300 cofactor, which may explain the reduced accumulation of lipids in white adipose cells. However, these findings suggest that increases in the thermogenic protein UCP1, CITED1, and CD137 during the ADMSC differentiation process may result from a reduction in pRb activity. These changes indicate that EID1 generates the beige phenotype during the process of differentiation of the multipotent cells used in this work (Zeve *et al.* 2009, Orbay *et al.* 2012). ADMSCs may express UCP1 after several days of treatment with rosiglitazone (Elabd *et al.* 2009). However, this study showed that rosiglitazone induced UCP1 expression during the differentiation process. However, transfection of EID1 resulted in significant differences in the levels of UCP1 protein.

Using an expression vector containing EID1 under the control of the aP2 promoter allowed the effects of EID1 on mature white adipocytes to be evaluated. The increased expression levels of thermogenic and mitochondrial proteins and of CITED1 suggest that EID1 may induce the beiging of white fat cells. The most likely mechanism of development of this beige phenotype is through an indirect increase in PGC1 α activity, followed by the inhibition of pRb. This latter protein plays an important role in the differentiation of mesenchymal stem cells;

thus, blocking its expression induces the activation of thermogenic proteins in different cell models (Scime *et al.* 2005, Calo *et al.* 2010, Hakim-Weber *et al.* 2011). Nevertheless, few descriptions of the pRb inhibition in differentiated adult human adipose cells are reported. Our group previously observed that EID1 binds and inhibits pRb in 3T3-L1 cells (Lizcano & Vargas 2010). Here, we demonstrated that EID1 reduced the expression of pRb in both ADMSCs and human differentiated adipocytes. Additionally, blockage of EID1 expression using a specific siRNA reduced PGC1 α expression, indicating a close functional relationship between these proteins. These findings indicate that the antagonism of EID1 toward pRb may indirectly increase the levels of PGC1 α . The aP2-EID1 expression vector may have therapeutic relevance because EID1 activity may be specific to white fat cells, and the metabolic effects of reducing pRb may benefit patients classified as obese (Hansen *et al.* 1999, Fajas *et al.* 2002). The observed effects of EID1 in adult human fat cells have not been previously described, with current data showing only phenotypic changes in animal models (Hansen *et al.* 2004a, Fernandez-Marcos & Auwerx 2010).

In conclusion, EID1 may act in two ways. First, it may modulate mesenchymal cells that are immersed in adipose tissue, affecting tissue metabolism and inducing the beige phenotype in these cells. Secondly, EID1 may play an important role in the transdifferentiation of white adipose cells to more metabolically active adipocytes, such as beige cells. Further research is necessary to determine the role of EID1 in regulating pRb protein and to assess any beneficial pharmacological effects resulting from the EID1 induction of thermogenic proteins, which may aid in the treatment of individuals with obesity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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