Dynamic Regulation of Genes Involved in Mitochondrial DNA Replication and Transcription during Mouse Brown Fat Cell Differentiation and Recruitment

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Abstract

Background: Brown adipocytes are specialised in dissipating energy through adaptive thermogenesis, whereas white adipocytes are specialised in energy storage. These essentially opposite functions are possible for two reasons relating to mitochondria, namely expression of uncoupling protein 1 (UCP1) and a remarkably higher mitochondrial abundance in brown adipocytes.

Methodology/Principal Findings: Here we report a comprehensive characterisation of gene expression linked to mitochondrial DNA replication, transcription and function during white and brown fat cell differentiation in vitro as well as in white and brown fat, brown adipose tissue fractions and in selected adipose tissues during cold exposure. We find a massive induction of the majority of such genes during brown adipocyte differentiation and recruitment, e.g. of the mitochondrial transcription factors A (Tfam) and B2 (Tfb2m), whereas only a subset of the same genes were induced during white adipose conversion. In addition, PR domain containing 16 (PRDM16) was found to be expressed at substantially higher levels in brown compared to white pre-adipocytes and adipocytes. We demonstrate that forced expression of Tfam but not Tfb2m in brown adipocyte precursor cells promotes mitochondrial DNA replication, and that silencing of PRDM16 expression during brown fat cell differentiation blunts mitochondrial biogenesis and expression of brown fat cell markers.

Conclusions/Significance: Using both in vitro and in vivo model systems of white and brown fat cell differentiation, we report a detailed characterisation of gene expression linked to mitochondrial biogenesis and function. We find significant differences in differentiating white and brown adipocytes, which might explain the notable increase in mitochondrial content observed during brown adipose conversion. In addition, our data support a key role of PRDM16 in triggering brown adipocyte differentiation, including mitochondrial biogenesis and expression of UCP1.

Citation: Murholm M, Dixen K, Qvortrup K, Hansen LHL, Amri E-Z, et al. (2009) Dynamic Regulation of Genes Involved in Mitochondrial DNA Replication and Transcription during Mouse Brown Fat Cell Differentiation and Recruitment. PLoS ONE 4(12): e8458. doi:10.1371/journal.pone.0008458

Editor: Patricia T. Bozza, Fundação Oswaldo Cruz, Brazil

Received 30 July 2009; Accepted 23 December 2009; Published 24 December 2009

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Funding: This work was supported by grants to J.B.H. from the Danish Medical Research Council, the Novo Nordisk Foundation, the Carlsberg Foundation, the Aase & Ejnar Danielsen Foundation, the Augustinus Foundation, the Beckett Foundation and Grosserer Ernst Fischers Mindelegat. M.M. was supported by the Novo Scholarship Program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

White and brown fat cells share a number of characteristics, including similarities in cell morphology, secretion of adipokines, enzymes of lipid metabolism and patterns of gene expression [1,2]. Despite these similarities, white and brown adipose tissues (WAT and BAT, respectively) carry out essentially opposite functions, with WAT being the major energy reserve through triglyceride accumulation, and BAT having the ability to dissipate energy through adaptive thermogenesis. The powerful energy dissipating and thermogenic capacity of BAT is due to two mitochondrial features of brown adipocytes: the presence of uncoupling protein 1 (UCP1) and a high number of mitochondria, which considerably exceeds that of white fat cells [1–3]. UCP1 is expressed exclusively in brown fat cells. Recently, it was demonstrated that white and brown fat cells do not develop from a common precursor, as BAT depots but not WAT seems to arise from precursor cells shared with skeletal muscle [4,5]. Intriguingly, brown-like adipocytes appearing in some WAT depots following extended periods of exposure to a β-adrenergic agonist do not share progenitors with skeletal muscle [4]. These observations suggest that two types of brown fat cells could exist in mammals.

The mitochondrial genome (mtDNA) is a circular, double-stranded molecule of 16,300 bp in mice. It is present in multiple copies in each mitochondrion and a mammalian cell contains between 1,000 and 10,000 copies [6–8]. mtDNA encodes key components of the electron transport chain as well as RNA components required for mitochondrial translation. The mitochondrial replication machinery is encoded by nuclear genes, the products of which translocate to the mitochondrion. These include
the mtDNA polymerase γ (Polg), the replicative mitochondrial helicase (Twinkle) and the mitochondrial RNA processing RNase (RNase MRP) [7].

Mitochondriogenesis is controlled by nucleus-encoded transcription regulators that localize to either the nucleus or the mitochondrion. Key among the former are the nuclear respiratory factor 1 (NRF-1) and NRF-2 [called GA repeat-binding protein (GABP) in the mouse], which control the expression of a large number of genes important for mitochondrial respiration and translation as well as mtDNA replication and transcription [6,8]. Nucleus-encoded transcription factors translocating to the mitochondrion include the mitochondrial RNA polymerase (PolRMT), mitochondrial transcription factors A, B1 and B2 (Tiam, Tfb1m, and Tb2m, respectively) and the mitochondrial transcription termination factor 1 (mTERF1) [7,9]. Three more mTERF genes have been identified (mTERF2-4) [10–12]. Finally, Tfam is not a termination factor 1 (mTERF1) [7,9]. Three more mTERF genes and Tfb2m, respectively) and the mitochondrial transcription factor 1 (mTERF1) [7,9]. Three more mTERF genes have been identified (mTERF2-4) [10–12]. Finally, Tfam is not only crucial for mitochondrial transcription, but is also a positive regulator of mtDNA copy number [13].

The regulation of mitochondrial biogenesis during adipocyte differentiation in general and the molecular background for the difference in abundance of mitochondria in white and brown fat cells is not fully understood, but the observation that levels of peroxisome proliferator-activated receptor γ (PPARγ) co-activator 1α (PGC-1α) and PGC-1β are substantially higher in the latter strongly suggest their involvement. This has subsequently been confirmed in brown adipocytes in vitro and in vivo, where it was found that simultaneous loss of PGC-1α and PGC-1β attenuates mitochondrial biogenesis, whereas loss of only one has little effect [14,15]. In addition, forced expression of PGC-1α in white adipocytes causes a transition towards a brown-like fat cell phenotype [16–18]. However, the mechanism by which PGC-1α and PGC-1β enforces mitochondrial biogenesis in brown fat cells is not fully explored.

Besides PGC-1α and PGC-1β, a number of nuclear transcription regulators have been reported to modulate mitochondrial biogenesis in adipocytes and/or adipose tissue, including PR domain containing 16 (PRDM16) [19], receptor interacting protein 140 (RIP140) [20], estrogen-related receptor α (ERRα) [21], small heterodimer partner (SHP) [22] and retinoblastoma protein (pRB) [23,24]. The effects of most of these have been linked directly or indirectly to the function of PGC-1 family members, e.g. PRDM16 that has been demonstrated to promote mitochondrial biogenesis and BAT-selective gene expression in adipocytes, at least in part by increasing activities of PGC-1α and PGC-1β, and additionally via direct binding and activation of the PGC-1α promoter [19,25].

Here we investigate the regulation of several aspects of mitochondrial function during adipocyte differentiation, with emphasis on brown adipogenesis in vitro and recruitment of brown fat cells in cold-challenged mice. We demonstrate that the majority of factors involved in mitochondrial transcription and replication are up-regulated during brown adipocyte conversion, whereas many of the same genes remain unchanged or are induced to a lesser extent during white adipocyte differentiation. In addition, we provide evidence that overexpression of Tiam or knockdown of PRDM16 influences mitochondrial DNA replication, gene expression and biogenesis.

Materials and Methods

Animals and Tissues

Interscapular BAT (iBAT) and epididymal WAT (eWAT) were obtained from a male C57BL/6J mouse. The stromal-vascular (SVF) and adipocyte (AF) fractions were obtained from twelve 10-week-old C57BL/6J mice (Charles River) that were maintained under constant temperature (21±2°C) and 12:12-h light-dark cycles with ad libitum access to standard chow diet and water. Animals were killed by cervical dislocation, and interscapular fat depots were rapidly excised and washed in phosphate-buffered saline, minced and digested for 45 min at 37°C with 2 mg/ml collagenase A (Roche) in the presence of 20 mg/ml bovine serum albumin (Sigma) in Dulbecco’s modified Eagle’s medium (DMEM) under mild agitation. After addition of fetal bovine serum (FBS), adipocytes were separated from stromal-vascular cells by filtration (250, 50 and 25 μm nylon filters) and centrifugation as described previously [26,27]. Contaminating erythrocytes were eliminated from the SVF by incubation in red blood cell lysis buffer (Sigma). Total RNA was extracted from each of the two pooled fractions as described below. For the cold-challenge experiment, female mice of the Sv129 strain were used as described [28,29]. Mice acclimated at 28°C were subsequently maintained at 28°C or placed at 6°C for 1 or 3 days, at which time interscapular and inguinal adipose tissues were dissected. Experimental groups included 4 mice. Animal procedures and care were in accordance with Italian National Institute of Medical Research guidelines approved by The European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

Cell Culture and Packaging of Virus

Wild-type and retinoblastoma gene-deficient (Rb−/−) mouse embryo fibroblasts (MEFs) were propagated and differentiated as previously described [30] with minor modifications. Briefly, MEFs were grown in AmnioMax C-100 Basal Medium (Invitrogen) supplemented with 7.5% FBS, 7.5% AmnioMax-C100 Supplement and 2 mM glutamine. The medium was changed every other day. For differentiation, 1-day postconfluent cells (designated day 0) were treated with growth medium containing 1 μM dexamethasone (Sigma), 0.5 mM methylisobutylxanthine and 0.5 mM rosiglitazone (kindly provided by Novo Nordisk) for 2 days. From day 2, the medium contained 5 μg/ml insulin (Roche) and 0.5 μM rosiglitazone and was changed every other day. The 3T3-L1 white pre-adipocyte cell line [31] was grown to confluence in DMEM containing 10% bovine serum. Two-days postconfluent cells (day 0) were induced to differentiate with growth medium containing 1 μM dexamethasone (Sigma), 0.5 mM methylisobutylxanthine (Sigma), 5 μg/ml insulin and 0.5 μM rosiglitazone. At days 2, 4 and 6, the cells were refed with DMEM containing 10% FBS supplemented with 5 μg/ml insulin and 0.5 μM rosiglitazone. The WT-1 pre-adipocyte cell line [32] was kindly provided by Dr. C. Ronald Kahn and was established by immortalisation of primary brown pre-adipocytes from newborn pups with simian virus 40 large T antigen. WT-1 cells were propagated and differentiated in DMEM supplemented with 10% FBS. For differentiation, 1-day postconfluent cells were treated with the same adiogenic compounds as described above for 3T3-L1 cells. Phoenix cells were cultured in DMEM with 10% FBS, and packaging and use of retrovirus were performed as previously described [33]. Transduced cells were selected with 8 μg/ml blasticidin S HCl (Invitrogen), 5 μg/ml puromycin (Sigma) or 200 μg/ml G418 (Sigma). All media described above contained 25% fetal bovine serum and 0.1% streptomycin, and all cells were cultured in a humidified atmosphere of 5% CO2 at 37°C.

Plasmids

The retroviral vectors pMSCVneo and pMSCVpuro were from Clontech and pMSCVbsd was kindly provided by Dr. Reuven Agami [34]. To improve the multiple cloning site (MCS) of
pMSCVbsd (and pMSCVpuro), the original HouseIII site between the PGK promoter and the blasticidin (or puromycin) resistance gene was destroyed and two partially annealed oligos (5'-GATCTGTATAAACCCGCACTGGGATCCAAGCT-3' and 5'-TCAAGGCGAGGCGGCCCAGCGC-3') were ligated into the BglII/Xhol site of the MCS, thereby creating pMSCVbsd link3 (or pMSCVpuro link3) having a new MCS with the following unique restriction enzyme sites: BglII-Pmel-SalI-VbnII-HindIII-MfeI-NdeI-ApaI-Xhol-HpaI-EcoRI. To improve the MCS of pMSCVneo, the original BamHI, SalI and HindIII sites between the neomycin resistance gene and the 3' long terminal repeat were destroyed and the two partially annealed oligos described above were ligated into the BglII/Xhol site of the MCS, thereby creating pMSCVneo link3 having a new MCS with the following unique restriction enzyme sites: EcoRI-Hpal-Xhol-ApaI-NdeI-SalI-HindIII-BamHI-Pmel-BglII. The pm-TFAM-HA vector encoding a HA-tagged full-length mouse Tpm1 was obtained from Dr. Dongchon Kang [35]. The insert was excised with BamHI/NdeI and ligated into the BamHI/NdeI site of pMSCVpuro link3 and pMSCVbsd link3, thereby creating the vectors pMSCVpuro-Tpm1-HA and pMSCVbsd-Tpm1-HA. The Thb2m-His-pBacPAK8 vector encoding full-length His-tagged mouse Thb2m was kindly provided by Dr. Claes M. Gustafsson [36]. The Thb2m insert was excised with XhoI/NdeI and ligated into the XhoI/NdeI site of pMSCVneo link3, thereby creating pMSCVneo- Thb2m-His. pSUPER.retro.neo was obtained from OligoEngine and pSUPER.retro.neo-PRDM16 has been described [19] and was purchased from Addgene (Addgene plasmid 15505).

**RT-qPCR**

Total RNA was purified from cells and tissues using TRIzol (Invitrogen) or Tri-Reagent (Euromedex). Reverse transcriptions were performed in 25 μl reactions containing 1x 1st Strand Buffer (Invitrogen), 2 μg random hexamers (GE Healthcare), 0.9 mM of each dNTP (GE Healthcare), 20 units of RNase-free RNase-free (GE Healthcare), 1 μg of total DNase-treated RNA and 200 units of Molenoy murine leukemia virus reverse transcriptase (Invitrogen). Reactions were left for 10 min at room temperature, followed by incubation at 37°C for 1 h. After cDNA synthesis, reactions were diluted with 50 μl of water and frozen at −80°C. cDNA was analysed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using the Strategene Mx3000P QPCR System. Each PCR mixture contained, in a final volume of 20 μl, 1.5 μl of each-strand cDNA, 10 μl of Brilliant QRT-PCR Master Mix (Strategene) and 2 pmol of each primer. PCR primers are listed in Table S1. All reactions were performed using the following cycling conditions: 95°C for 10 min, then 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 15 s. PCR was carried out in 96-well plates and each sample was run in duplicate. Target gene mRNA expression was normalized to expression of TATA-binding protein (TBP) mRNA or 18S ribosomal RNA (rRNA). Absolute values of samples normalised to TBP (gene of interest/TBP) are provided in Table S2 to allow comparison of expression levels across cell lines and tissues.

**Quantification of Relative mtDNA Copy Numbers**

Cells were washed in phosphate-buffered saline, scraped off the plates in lysis buffer containing 100 mM Tris-base (pH 8.0), 5 mM EDTA (pH 8.0), 0.2% sodium dodecyl sulphate, 200 mM NaCl and 100 μg/ml proteinase K and incubated overnight at 55°C with rotation. DNA was precipitated with two volumes of 99% ethanol and fished out with inoculation loops, washed in 70% ethanol and dissolved in 1xTE buffer containing 10 μg/ml RNase A at 55°C overnight. Two dishes were harvested at each time point. DNA concentrations were determined on the Eppendorf BioPhotometer at 260 nm and 50 ng DNA was used for qPCR. Primers sets used were against COX II (mtDNA) and RIP140 (nuclear DNA) (Table S1).

**Citrate Synthase Activity**

Cells were harvested in GG-buffer (pH 7.5) containing 25 mM glycyglycine, 150 mM KCl, 5 mM MgSO4 and 5 mM EDTA as well as freshly added DTT (1 mM), BSA (0.02%) and Triton X-100 (0.1%), vortexed and frozen in liquid nitrogen. Two dishes were harvested at each time point. Samples were thawed on ice and centrifuged at 4°C at 20,000 g for 2 minutes. Supernatants were used for measurements. Citrate synthase activity was measured spectrophotometrically at 25°C and 412 nm. Citrate synthase buffer contained 100 mM Tris-base (pH 8.0), 10 mM DTNB, 5 mM acetyl-CoA and 50 mM oxaloacetic acid [37]. Each sample was measured in duplicate and the mean was used for subsequent calculations. Activities were normalized to the amount of protein determined by the Lowry method [38].

**Transmission Electron Microscopy**

Cells were washed in 37°C 0.15 M Sorensens Phosphate Buffer (pH 7.4) (Electron Microscopy Sciences) and subsequently fixed in 2% glutaraldehyde in 0.05 M Sorensens Phosphate Buffer while rotating overnight at 4°C. The samples were rinsed three times in 0.15 M Sorensens Phosphate Buffer (pH 7.4) and subsequently postfixed in 1% OsO4 in 0.12 M sodium cacodylate buffer (pH 7.4) for 2 h. The specimens were dehydrated ingraded series of ethanol, transferred to propylene oxide and embedded in Epon according to standard procedures. Ultra thin sections were cut with a Reichert-Jung Ultracut E microtome and collected on single slot copper grids with Formvar supporting membranes. The sections were stained with uranyl acetate and lead citrate and examined with a Philips CM 100 transmission electron microscope, operated at an accelerating voltage of 80 kV and equipped with a SIS MegaView2 camera. Digital images were recorded with the analysIS software package.

**Statistical Analyses**

Time-course studies with wild-type and Rb−/− MEFs were analysed for statistical significance (p<0.05) using multiple linear regression of means using PROC REG (SAS 9.1.2, SAS Institute) with expression level as the dependent variable and cell type and time as independent variables. It was assumed that residual variance was identical for the two cell types. A difference between means was considered statistically significant if there was no overlap between their 95% confidence intervals. All other relevant data were analysed for statistical significance (p<0.05) using Student’s t-test (Microsoft Office Excel). Statistical analysis of data for eWAT and iBAT was not possible, as the tissues were from a single mouse. Similarly, statistics were not conducted on BAT fractions, as the measurements were performed on pools of RNA from 12 mice.

**Results**

**Expression of Brown Adipose-Selective Genes during Differentiation of White and Brown Adipocytes**

We aimed at characterising various aspects of mitochondrial biogenesis and function during fat cell differentiation, with emphasis on differences between white and brown adipocytes. Therefore, we initially measured expression levels of genes involved in mitochondrial DNA replication, transcription and function during adipogenesis of mouse fibroblasts and pre-adipocytes capable of differentiation to white or brown fat cells. We reported previously that wild-type mouse embryo fibroblasts (MEFs) differentiate to white fat cells, whereas MEFs lacking a functional retinoblastoma
gene (designated Rb−/− MEFs) differentiate to brown adipocytes [23]. Wild-type and Rb−/− MEFs were subjected to a time-course study. In addition, we used two pre-adipocyte cell lines, namely 3T3-L1 white and WT-1 brown pre-adipocytes as supplemental model systems to validate expression patterns of selected genes. Gene expression was determined by quantitative RT-PCR (RT-qPCR) and presented as indicated in the figure legends. Table S2 provides the normalised expression of all genes measured to allow comparison of expression levels across cell lines and tissues. Wild-type MEFs, Rb−/− MEFs, 3T3-L1 and WT-1 cells efficiently differentiated to adipocytes following adipogenic stimulation at day 0, as demonstrated by robust induction of adipocyte marker genes like PPARγ2, CCAAT/enhancer-binding protein α (C/EBPα), adiponectin and fatty acid-binding protein 4 (FABP4, also called aP2) (as determined by RT-qPCR) as well as accumulation of lipid droplets in >90% of the cells (Fig. S1 and data not shown). Cells were considered as mature adipocytes at day 8. The general marker genes were expressed at equal levels in epididymal WAT (eWAT) and interscapular BAT (iBAT), except for adiponectin, which was enriched in the former (Fig. S1). eWAT was chosen as WAT depot in this case, as it contains very few brown adipocytes and is refractory to cold-induced recruitment of brown-like adipocytes [39]. Conversely, brown adipose-specific or -enriched genes like UCP1, cell death-inducing DFF45-like effector A (Cidea) [40] and carnitine palmitoyltransferase 1b (CPT-1b) were selectively induced in differentiating Rb−/− MEFs and WT-1 cells compared to differentiating wild-type MEFs and 3T3-L1 pre-adipocytes (Fig. 1A). Levels of UCP1 mRNA in Rb−/− and WT-1 adipocytes were 5–10 times lower than in iBAT, but 3000–6000 times higher than in eWAT (Table S2). Most markers, whether general adipose markers or brown fat-enriched markers, started to increase around day 2 or 3. To probe whether the cell lines becoming brown adipocytes, like primary brown pre-adipocytes [4,5], expressed markers of skeletal muscle before differentiation, we measured the level of selected skeletal muscle markers at day 0. Consistent with their brown adipogenic fate, myogenin mRNA was present at higher levels in Rb−/− and WT-1 cells compared to wild-type MEFs and 3T3-L1 cells (Fig. S2). Of interest, myogenin was expressed at substantially higher levels in WT-1 pre-adipocytes compared to Rb−/− MEFs (Table S2), possibly reflecting their different origins as primary brown pre-adipocytes and embryo fibroblasts, respectively. Of notice, expression of MyoD and Myf-5 was not enriched in Rb−/− MEFs and WT-1 pre-adipocytes (data not shown).

Figure 1. Expression of brown fat-selective genes and genes indicative of differential mitochondrial biogenesis during differentiation of white and brown adipocytes. Cell lines were induced to differentiate as described in “Materials and Methods” and total RNA was harvested at the indicated days of differentiation. In addition, RNA from eWAT and iBAT was included. Expression levels were determined by RT-qPCR and relative expression levels of genes indicated in the figure determined by normalisation to the levels of TBP. In each of the three boxes for the individual genes, the mean of the normalized expression level of the sample with the highest value was set to 100. Error bars represent SEM. (A) Expression of the brown adipose-selective genes UCP1, Cidea and CPT-1b. (B) Gene expression pattern indicative of differential mitochondrial biogenesis. Genes measured were CS, Cyc1 and COX II. Results from one of two independent cell culture experiments are shown. *, p<0.05 (day X in wild-type MEFs (or 3T3-L1) compared to day X in Rb−/− MEFs (or WT-1)). ▲, p<0.05 (day 0 vs. day 8 for each of the four cell lines). doi:10.1371/journal.pone.0008458.g001
Changes in Gene Expression, Enzyme Activities and mtDNA Copy Number Indicative of Differential Mitochondrial Biogenesis during Differentiation of White and Brown Fat Cells In Vitro

Citrate synthase (CS) has been used in adipose tissue as a measure of mitochondrial respiratory chain activity and mitochondrial biogenesis [41,42]. CS mRNA levels increase during differentiation of all four differentiation models used here, but are induced to 3–4-fold higher levels in Rb−/− and WT-1 cells compared to wild-type MEFs and 3T3-L1 cells (Fig. 1B). Similarly, CS enzyme activity was increased during adipose conversion of both wild-type and Rb−/− MEFs, with CS reaching a higher activity in the latter (Fig. 2A). Of notice, the difference in CS activity between wild-type and Rb−/− fat cells was smaller than the difference observed for CS mRNA levels. Expression of two electron transport chain components, cytochrome c-1 (Cyc1) and cytochrome c oxidase II (COX II), was induced to ∼4-fold higher levels in differentiating Rb−/− compared with wild-type MEFs (Fig. 1B). Cyc1 displayed a similar fold induction during differentiation of WT-1 and 3T3-L1 cells, although the absolute level was ∼2-fold higher in the former (Fig. 1B). COX II was induced ∼6-fold in WT-1 and ∼2-fold in 3T3-L1 cells. Consistently, CS, Cyc1 and COX II mRNAs were enriched in iBAT relative to eWAT. To probe whether the increased level and activity of CS were accompanied by changes in mtDNA copy number, we determined the ratio of mtDNA and nuclear DNA (nDNA) by qPCR before differentiation was initiated (day 0) and in the mature adipose state (day 8). The mtDNA/nDNA ratio increased 3.5-fold in wild-type cells and 7-fold in Rb−/− cells, suggesting that more mtDNA replication occurred during brown compared with white adipose conversion (Fig. 2B). In summary, the gene expression pattern in Rb−/− and WT-1 brown adipocytes indicates a more pronounced mitochondrial biogenesis than in wild-type MEF-derived and 3T3-L1 white adipocytes.

Transmission Electron Microscopy Demonstrates Mitochondrial Biogenesis during Adipose Conversion of White and Brown Fat Cells In Vitro

To obtain morphological evidence for mitochondrial biogenesis during adipocyte differentiation, wild-type and Rb−/−
MEFs were investigated by electron microscopy at days 0 and 8. Confluent fibroblasts (day 0) contain relatively few mitochondria, irrespectively of Rb status (Fig. 3). Consistent with the gene expression profiles and mtDNA levels described above, mitochondrial numbers seemed to increase during differentiation of both wild-type and Rb−/− cells, and mitochondrial density appeared substantially higher in mature Rb−/− brown adipocytes (Fig. 3).

Expression of Genes Involved in mtDNA Replication during Differentiation of White and Brown Fat Cells In Vitro

To delineate how expression of mitochondrial factors involved in replication and transcription of mtDNA was regulated, we measured their levels in the four cell lines. The functional mtDNA polymerase γ consists of a catalytic subunit (Polg-A) in complex with two copies of the accessory subunit Polg-B. Expression of the Polg subunits, Ssb, Twinkle and RNase MRP was higher in iBAT.

Figure 3. Transmission electron micrographs of cells before and after differentiation. Wild-type and Rb−/− MEFs were harvested for transmission electron microscopy at days 0 and 8 of differentiation as described in “Materials and Methods”. Two representative cells of each cell line are shown at each time point. Similar results were obtained in two independent experiments.

doi:10.1371/journal.pone.0008458.g003
Expression of Genes Involved in Transcription of mtDNA during Differentiation of White and Brown Fat Cells In Vitro

PolRMT is unable to interact with promoter sequences unless assisted by Tbam and either Tfb1m or Tfb2m [7,9]. mTERF1, and potentially also mTERF2-4, is involved in termination of mtDNA transcripts, but has also recently been suggested to promote transcription initiation. PolRMT was induced early during adipose conversion of wild-type and Rh−/− MEFs, with levels increasing at day 1, and levels in Rh−/− cells exceeding that of wild-type cells by day 2 [Fig. 4B]. Similarly, PolRMT was induced during adipose conversion of both 3T3-L1 and WT-1 cells. Expression of Tbam and Tfb2m was relatively constant in differentiating wild-type cells, whereas both increased ~4-fold in Rh−/− cells [Fig. 4B]. Tfb1m expression was induced already at day 1 and continued to increase until day 4 or 5, after which expression was stable (wild-type cells) or decreased (Rh−/− cells) [Fig. 4B]. The level of Tfb1m was moderately higher after day 2 in Rh−/− compared with wild-type cells. Tbam and Tfb2m were both induced ~2-fold during differentiation of 3T3-L1 and ~3-fold during differentiation of WT-1 cells [Fig. 4B]. In contrast, Tfb1m was induced ~4-fold and ~3-fold during differentiation of 3T3-L1 and WT-1 cells, respectively. The mitochondrial ribosomal protein L12 (Mrpl12) has been demonstrated to enhance mtDNA transcription via physical interaction with PolRMT [13]. Mrpl12 was induced ~5-fold and ~20-fold during differentiation of wild-type and Rh−/− MEFs, respectively, with expression beginning to increase earlier in the latter [Fig. S3]. Expression of Mrpl12 was strongly induced during adipose conversion of both 3T3-L1 and WT-1 cells. Fluctuations of the mTERF1 and mTERF3 mRNAs were observed during differentiation of MEFs, with levels being moderately higher in Rh−/− relative to wild-type mature adipocytes [Fig. S3]. mTERF1 was slightly down-regulated during differentiation of 3T3-L1 cells, whereas it was moderately induced during WT-1 adipogenesis [Fig. S3]. mTERF2 was transiently down-regulated in differentiating MEFs and expression levels were ~2-fold higher in Rh−/− relative to wild-type cells on most days [Fig. S3]. During differentiation of MEFs, mTERF4 was selectively induced in Rh−/− cells. mTERF2, mTERF3 and mTERF4 were induced 1.5-3-fold during differentiation of both 3T3-L1 and WT-1 cells [Fig. S3]. PolRMT, Tbam, Tfb1m, Tfb2m, Mrpl12, mTERF2, mTERF3 and mTERF4 were enriched in iBAT relative to eWAT, whereas mTERF1 was expressed at similar levels [Fig. 4B and S3].

Expression of Nuclear DNA-Binding Transcription Factors Linked to Mitochondrial Biogenesis and Function during Differentiation of White and Brown Fat Cells In Vitro

Expression of NRF-1 was relatively constant during white adipocyte differentiation of wild-type MEFs and was induced 1.7-fold at late stages of brown adipogenesis of Rh−/− MEFs [Fig. 5B]. In 3T3-L1 and WT-1 cells, NRF-1 was moderately down- and up-regulated, respectively. NRF-1 was expressed at moderately (1.6-fold) higher levels in iBAT relative to eWAT. GABPβ and GABPβ displayed a similar expression pattern during differentiation of white and brown fat cells, with a transient down-regulation at days 1 and 2 (except for GABPβ in Rh−/− cells) as well as a second down-regulation at the terminal stages of differentiation (days 6 and 8) [Fig. 5B]. In addition, whereas GABPβ was expressed at similar levels in wild-type and Rh−/− cells during differentiation, GABPβ was moderately higher on most days in Rh−/− cells. GABPβ was induced nearly 2-fold during brown adipose conversion of WT-1 cells, whereas its expression was unchanged from day 0 to day 8 in 3T3-L1 cells. In differentiating 3T3-L1 and WT-1 cells, GABPβ was moderately down-regulated. In eWAT and iBAT, GABPβ and GABPβ were expressed at ~2-fold higher levels in the latter [Fig. 5B]. Of interest, PRDM16 was expressed at dramatically higher levels in
Figure 4. Expression of genes involved in mtDNA replication and transcription during differentiation of white and brown adipocytes. Cell lines were induced to differentiate as described in “Materials and Methods” and total RNA was harvested at the indicated days of differentiation. In addition, RNA from eWAT and iBAT was included. Expression levels were determined by RT-qPCR and relative expression levels of genes indicated in the figure determined by normalisation to the levels of TBP. In each of the three boxes for the individual genes, the mean of the normalized expression level of the sample with the highest value was set to 100. Error bars represent SEM. (A) Expression of genes involved in mtDNA replication. Genes measured were Polg-A, Polg-B, Ssb, Twinkle, RNase MRP and RNase MRP RNA. (B) Expression of genes involved in mitochondrial transcription. Genes measured were PolRMT, Tfam, Tfb1m and Tfb2m. Results from one of two independent cell culture experiments are shown. *, p<0.05 (day X in wild-type MEFs (or 3T3-L1) compared to day X in Rb−/− MEFs (or WT-1)). ▲, p<0.05 (day 0 vs. day 8 for each of the four cell lines).

doi:10.1371/journal.pone.0008458.g004
Figure 5. Expression of nuclear co-regulators and DNA-binding transcription factors linked to mitochondrial biogenesis and function during differentiation of white and brown fat cells. Cell lines were induced to differentiate as described in "Materials and Methods" and total RNA was harvested at the indicated days of differentiation. In addition, RNA from eWAT and iBAT was included. Expression levels were determined by RT-qPCR and relative expression levels of genes indicated in the figure determined by normalisation to the levels of TBP. In each of the three boxes for the individual genes, the mean of the normalized expression level of the sample with the highest value was set to 100. Error bars represent SEM. (A) Expression of PGC-1 family members and RIP140. (B) Expression of the nuclear DNA-binding transcription factors NRF-1, GABPA, GABPB, PRDM16, PPARα and ERRα. Results from one of two independent cell culture experiments are shown. *, p<0.05 [day X in wild-type MEFs (or 3T3-L1) compared to day X in Rb2/2 MEFs (or WT-1)]. ▲, p<0.05 (day 0 vs. day 8 for each of the four cell lines).

doi:10.1371/journal.pone.0008458.g005
MEFs, but ERRα, the transcription of Rbα fold) even before differentiation was initiated (day 0). In addition, wild-type and 3T3-L1 fat cells (Fig. 5B). Expression of ERRα adipose conversion of all cell types, but were induced to much higher levels in Rbα−/− and WT-1 adipocytes compared with wild-type and 3T3-L1 fat cells (Fig. 5B). Expression of ERRα was induced ~6-fold during differentiation of wild-type and Rbα−/− MEFs, but ERRα levels were substantially higher in mature Rbα−/− relative to wild-type adipocytes. In 3T3-L1 and WT-1 cells, ERRα was induced ~6-~12-fold during differentiation (Fig. 5B). Both PPARγ and ERRα were enriched in iBAT relative to eWAT, consistent with previous findings [48–50]. As SHP-deficiency has been shown to increase expression of PGC-1α and UCP1 as well as mitochondrial biogenesis [22], we also measured the expression of SHP. Consistent with previously reported data [51] (www.nursa.org/10.1621/datasets.01006; www.nursa.org/10.1621/datasets.020001), we failed to reproducibly detect expression of SHP in eWAT and iBAT as well as in the cell lines used in this study (data not shown).

### Discussion

The capacity of brown fat cells to dissipate significant amounts of energy as heat requires a high mitochondrial density and a mechanism of uncoupling oxidative phosphorylation. The former requirement is met by a mitochondrial volume fraction of BAT of approximately 30% [54], and the latter by the unique presence of UCP1 at high levels in the mitochondrial inner membrane. In the present report, we provide a detailed analysis of gene expression linked to replication and transcription of mtDNA as well as expression profiles of nuclear transcription regulators of mitochondrial function during white and brown fat cell differentiation in vitro and in adipose tissues from cold-challenged mice.
Adipocyte differentiation is associated with mitochondrial biogenesis [55]. Corroborating this, we measured a substantial induction of CS expression and mtDNA copy number during differentiation of both white and brown adipocytes, with induction being particularly pronounced during brown adipogenesis (Fig. 1B). Several genes encoding transcription regulators of mitochondrial function were differently regulated during white and brown fat cell differentiation in vitro. Of those acting in the

| Figure 6. Expression of selected genes in brown adipose tissue fractions and adipose tissues from cold-challenged mice. | RNA from BAT fractions (pool from 12 mice) and adipose tissues from cold-challenged mice (4 animals in each group) was analysed by RT-qPCR. Data for SVF and AF samples from BAT are shown in the left box, whereas data for samples from iBAT and iWAT of cold-challenged mice are shown in the right box. Expression levels in BAT fractions were normalised to the expression of 18S rRNA. Expression levels in adipose tissue from cold-challenged mice were normalised to the expression of TBP. In each of the two boxes for the individual genes, the mean of the normalized expression level of the sample with the highest value was set to 100. Error bars represent SEM. (A) Expression of UCP1, PGC-1α, PRDM16, CPT-1b and CS. (B) Expression of Tfam, Tfb1m and Tfb2m. *, p < 0.05 [day X at 28 °C in iBAT (or iWAT) compared to day X at 6 °C in iBAT (or iWAT)].
doi:10.1371/journal.pone.0008458.g006

| Table 1. Summary of gene expression changes in brown adipose tissue fractions and adipose tissues from cold-challenged mice. |
| --- | --- | --- | --- | --- | --- |
| SVF AF | iBAT | iWAT | iBAT | iWAT |
| UCP1 |  |  |  |  |
| PGC-1α |  |  |  |  |
| PRDM16 |  |  |  |  |
| CPT-1b |  |  |  |  |
| CS |  |  |  |  |
| Tfam |  |  |  |  |
| Tfb1m |  |  |  |  |
| Tfb2m |  |  |  |  |

| Figure 6. | Expression of selected genes in brown adipose tissue fractions and adipose tissues from cold-challenged mice. | RNA from BAT fractions (pool from 12 mice) and adipose tissues from cold-challenged mice (4 animals in each group) was analysed by RT-qPCR. Data for SVF and AF samples from BAT are shown in the left box, whereas data for samples from iBAT and iWAT of cold-challenged mice are shown in the right box. Expression levels in BAT fractions were normalised to the expression of 18S rRNA. Expression levels in adipose tissue from cold-challenged mice were normalised to the expression of TBP. In each of the two boxes for the individual genes, the mean of the normalized expression level of the sample with the highest value was set to 100. Error bars represent SEM. (A) Expression of UCP1, PGC-1α, PRDM16, CPT-1b and CS. (B) Expression of Tfam, Tfb1m and Tfb2m. *, p < 0.05 [day X at 28 °C in iBAT (or iWAT) compared to day X at 6 °C in iBAT (or iWAT)].
doi:10.1371/journal.pone.0008458.g006
nucleus, PRDM16, NRF-1, GABPα, PPARα and PGC-1α were expressed or induced preferentially during brown adipose conversion, whereas the co-repressor RIP140 was induced primarily during white fat cell differentiation. Tfam and Tfb2m were selectively induced in differentiating Rb−/− MEFS compared to wild-type MEFS and elicited a more pronounced induction during differentiation of WT-1 relative to 3T3-L1 pre-adipocytes.

Tfam is a key regulator of mtDNA transcription, mtDNA copy number and mitochondrial biogenesis [13,56,57]. Moreover, Tfb2m promotes mtDNA transcription more efficiently than Tfb1m [58–60]. As mentioned above, expression of Tfam, Tfb1m

Figure 7. Effect of forced expression of Tfam and Tfb2m on mtDNA replication and citrate synthase expression. Rb−/− MEFS were transduced with an empty retrovirus (designated "Vector") or retroviral vectors encoding Tfam or Tfb2m. Total DNA and RNA were harvested at days 0 and 8. (A) Relative mtDNA copy number was determined by qPCR using primer sets specific for mtDNA (COX II) and nDNA (RIP140) and relative mtDNA levels calculated by normalising signals from COX II to those of RIP140. (B) Expression levels were determined by RT-qPCR and the relative expression level of CS determined by normalisation to the level of TBP. The results presented are the means from three independent experiments. The Vector sample on day 0 was set to 1 in each experiment. Error bars represent SEM. *, p<0.05 (day X in Vector compared to day X in Tfam or Tfb2m). ▲, p<0.05 (day 0 vs. day 8 for Vector, Tfam or Tfb2m).

doi:10.1371/journal.pone.0008458.g007

Figure 8. Effect of silencing of PRDM16 expression on expression levels of mitochondrial marker genes. Rb−/− MEFS were transduced with pSUPER.retro.neo or pSUPER.retro.neo-PRDM16 virus (designated "Vector" and "shPRDM16", respectively), selected, replated and induced to differentiate as described in "Materials and Methods" and total RNA was harvested at days 0 and 8 of differentiation. Expression levels were determined by RT-qPCR and relative expression levels of genes indicated in the figure determined by normalisation to the levels of TBP. In each of the boxes for the individual genes, the mean of the normalized expression level of the sample with the highest value was set to 100. Error bars represent SEM. Shown is the expression of PRDM16, PGC-1α, UCP1, CPT-1b and CS. A representative of three independent experiments is shown. *, p<0.05 (day X in Vector compared to day X in shPRDM16). ▲, p<0.05 (day 0 vs. day 8 for Vector or shPRDM16).

doi:10.1371/journal.pone.0008458.g008
PRDM16 expression is induced in both iBAT and iWAT (Fig. 6A). BAT relative to SVF from WAT. In cold-challenged mice, PRDM16 is strongly enriched in iBAT relative to eWAT and is expressed at dramatically higher levels in both the undifferentiated (2-fold) and differentiated state (1.5-fold), suggesting that Tiam promotes mitochondrial biogenesis in these cells. The doubling of mtDNA on day 0 by Tiam overexpression is relatively minor compared to the 7-fold increase observed during differentiation of Rb2/−/− cells (Fig. 2B). This suggests that Tiam alone is unable to drive the level of mitochondrial biogenesis observed during brown adipose conversion and that additional factors are required. Such additional factors might be any of the nuclear transcription factors involved in mitochondrial function or any of the mitochondrial transcription or replication factors. Nevertheless, our expression profiles support a role of Tiam and Tfb2m in controlling the differential mtDNA replication and mitochondrial biogenesis during white and brown adipocyte differentiation. Finally, the induction of Tiam and Tfb2m in iWAT following cold exposure suggests their involvement in the mitochondrial biogenesis taking place (as indicated by the induction of CS) during the transformation to a BAT-like depot. Decisive evidence for a functional role of Tiam, Tfb1m and Tfb2m in mitochondrial biogenesis and function in adipose tissue awaits the generation of tissue-specific knockout mice.

The identification of the causative signal that triggers mitochondrial biogenesis and UCP1 expression during brown adipose conversion is key to establishing the molecular background for the differential metabolic functions of white and brown fat. PGC-1α and PGC-1β are crucial in brown adipogenesis, being necessary for both mitochondrial biogenesis and UCP1 expression in vitro and in vivo. However, these effects require that both factors are absent simultaneously, as absence of either PGC-1α or PGC-1β has little effect [14,15]. Therefore, PGC-1α or PGC-1β is unlikely to be the triggering factor that causes functional brown adipocyte differentiation. We find that PGC-1α is not enriched in precursor cells (day 0) destined to become brown fat cells (Rb2/−/− MEFs and WT-1 pre-adipocytes) relative to those destined to become white adipocytes (wild-type MEFs and 3T3-L1 pre-adipocytes). However, PGC-1α is induced as early as day 1 in Rb2/−/− cells, whereas PGC-1β is induced one day later. Overall, our expression profiles support an important role of PGC-1α and PGC-1β in brown adipocyte differentiation and function, but not as triggering factors. Contrary, PRDM16 is expressed at dramatically higher levels in Rb2/−/− relative to wild-type MEFs and in WT-1 relative to 3T3-L1 pre-adipocytes prior to differentiation (Fig. 4B). In exponentially growing cultures of wild-type MEFs, we failed to detect expression of PRDM16, whereas it was easily detectable in proliferating Rb2/−/− MEFs (data not shown). PRDM16 is strongly enriched in iBAT relative to eWAT and is expressed at higher levels in brown AF compared to SVF (Fig. 6A) [19], but it has not been reported whether PRDM16 is enriched in SVF from BAT relative to SVF from WAT. In cold-challenged mice, PRDM16 expression is induced in both iBAT and iWAT (Fig. 6A). Based on the powerful effect of PRDM16 on brown adipose conversion of white pre-adipocytes and myoblasts as well as its reported expression patterns, PRDM16 is a prime candidate as priming and triggering factor for brown adipocyte differentiation. Consistent with previous reports [4,19,25], silencing of PRDM16 in brown adipocyte precursor cells (in this case Rb2/−/− MEFs) blunted the induction of typical brown fat marker genes, like UCP1, CPT-1b and PGC-1α (Fig. 8). Moreover, induction of CS expression was reduced in PRDM16 knockdown cells, suggesting that mitochondrial biogenesis was diminished. However, mitochondrial transcription and replication factors were not substantially affected. The reason for the relatively minor effect of PRDM16 knockdown on CS expression and the lack of effect on induction of mitochondrial factors might be ascribed to a knockdown of only ∼3-6-fold, which means that the remaining amount of PRDM16 mRNA still exceeds the amount present in the corresponding wild-type cells (see Fig. 5B). It will be relevant to clarify how expression of PRDM16 is regulated, as little is known about signalling pathways and transcription factors impacting on the PRDM16 promoter. However, the observation that PRDM16 is enriched in cells with the capacity to differentiate to brown adipocytes relative to comparable cells with the capacity to become white fat cells, even before adipogenesis is induced, suggests that the PRDM16 gene is subject to differential epigenetic regulation in white and brown pre-adipocytes.

Recent findings demonstrate the existence of active BAT in a subset of adult humans [61–66]. Based on the anti-obesity function of BAT in rodents, a better understanding of the proliferative and thermogenic potential of this tissue is of significant interest. As mitochondria are important for BAT function, detailed information on processes leading to mitochondrial biogenesis in adipocytes is of potential relevance for the development of future anti-obesity regimens.

Here we have identified a highly dynamic pattern of expression of genes involved in replication and transcription of mtDNA as well as of nuclear transcription factors regulating mitochondrial function during white and brown adipocyte differentiation. Specific or selective induction of a number of these was observed during brown adipose conversion, including PGC-1α, Tiam and Tfb2m. PRDM16 was expressed at higher levels in brown compared to white adipocyte precursor cells. We provide evidence that modulation of Tiam and PRDM16 levels affect mitochondrial DNA replication, gene expression and biogenesis during fat cell differentiation. In summary, the molecular machinery controlling mitochondrial biogenesis and function is differentially regulated during white and brown adipocyte differentiation, and our data are consistent with a key role of PRDM16 in priming and triggering brown adipogenesis.

**Supporting Information**

**Figure S1** Expression of general adipose markers during differentiation of white and brown fat cells. Cell lines were induced to differentiate as described in “Materials and Methods” and total RNA was harvested at the indicated days of differentiation. In addition, RNA from eWAT and iBAT was included. Expression levels were determined by RT-qPCR and relative expression levels of genes indicated in the figure determined by normalisation to the levels of TBP. In each of the three boxes for the individual genes, the mean of the normalized expression level of the sample with the highest value was set to 100. Error bars represent SEM. Genes measured were PPARG, C/EBPβ, FABP4 and adiponectin. Results from one of two independent cell culture experiments are shown. *, p<0.05 [day X in wild-type MEFs (or 3T3-L1) compared to day X in Rb2/−/− MEFs (or WT-1)]. Δ, p<0.05 [day 0 vs. day 8 for each of the four cell lines].

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**PLoS ONE | www.plosone.org 13 December 2009 | Volume 4 | Issue 12 | e8458**
**Figure S2** Expression of myogenin in white and brown adipocyte precursor cells. Total RNA was harvested at day 0 and relative expression of myogenin was determined by RT-qPCR by normalisation to TBP. In each of the two boxes, the mean of the normalized expression level of the sample with the highest value was set to 100. Error bars represent SEM. Results from one of two independent experiments are shown. *, p<0.05 [wild-type MEFs (or 3T3-L1) compared to Rb<–/– MEFs (or WT-1)].

**Figure S3** Expression of genes involved in mitochondrial transcription during differentiation of white and brown adipocytes. Cell lines were induced to differentiate as described in “Materials and Methods” and total RNA was harvested at the indicated days of differentiation. In addition, RNA from eWAT and iBAT was included. Expression levels were determined by RT-qPCR and relative expression levels of genes indicated in the figure determined by normalisation to the levels of TBP. In each of the three boxes for the individual genes, the mean of the normalized expression level of the sample with the highest value was set to 100. Error bars represent SEM. Genes measured were Mrpl12 and mTERF1-4. Results from one of two independent cell culture experiments are shown. *, p<0.05 [day X in wild-type MEFs (or 3T3-L1) compared to day X in Rb<–/– MEFs (or WT-1)]. ∆, p<0.05 (day 0 vs. day 8 for each of the four cell lines).

**Figure S4** Expression of PPARγ2 and adiponectin in stromal-vascular and adipose fractions of brown adipose tissue. RNA from BAT fractions (pool of 12 mice) was analysed for the expression of PPARγ2 and adiponectin by RT-qPCR. Expression levels were normalised to the expression of 18S rRNA. The highest value of the mean of the normalized expression levels of PPARγ2 or adiponectin was set to 100.

**Figure S5** Expression of selected transcription factors after silencing of PRDM16 expression. Rb<–/– MEFs were transduced with pSUPER.retro.neo or pSUPER.retro.neo-PRDM16 virus (designated “Vector” and “shPRDM16”, respectively), selected, replated and induced to differentiate as described in “Materials and Methods” and total RNA was harvested at days 0 and 8 of differentiation. Expression levels were determined by RT-qPCR and relative expression levels of genes indicated in the figure determined by normalisation to the levels of TBP. In each of the boxes for the individual genes, the mean of the normalized expression level of the sample with the highest value was set to 100. Error bars represent SEM. (A) Expression of PPARγ2, adiponectin, FABP4 and myogenin. (B) Expression of Tfam, Tbk1m, Tbkm2 and GABPF-2. Similar results were obtained in three independent experiments. Similar results were obtained in three independent experiments. *, p<0.05 (day X in Vector compared to day X in shPRDM16). ∆, p<0.05 (day 0 vs. day 8 for Vector or shPRDM16).

**Table S1** Primers used for quantitative PCR

**Table S2** Normalised expression levels of analysed genes. To allow comparison of expression levels across cell lines and tissues, relevant samples normalised to TBP (gene of interest/TBP) are listed. Expression levels for BAT fractions are not provided, as normalisation in this case was to 18S rRNA.

**Acknowledgments**

We thank Dr. C. Ronald Kahn (Joslin Diabetes Center and Harvard Medical School, Boston) for the generous gift of WT-1 cells and Drs. Dongshen Kang (Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan), Claes M. Gustafsson (Karolinska Institutet, Stockholm, Sweden) and Reuven Agami (The Netherlands Cancer Institute, Amsterdam, the Netherlands) for valuable plasmids. We are grateful to Mary-Anne Gleie for technical assistance. We thank Novo Nordisk for the kind gift of rosiglitazone.

**Author Contributions**

Conceived and designed the experiments: MM KD GB BQ JBH. Performed the experiments: MM KD KQ LHJF EZA LM GB JBH. Analyzed the data: MM KD KQ JBH. Wrote the paper: JBH.

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