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Atrophy of brown adipocytes in the adult mouse causes transformation into white adipocyte-like cells

Dae Whan Kim[1]*, Beom Sue Kim[1]* Hee-Seok Kwon[1], Chan Gil Kim[2] Han-Woong Lee[3], Woong Hwan Choi[4] and Chul Geun Kim[1,5]

[1]The Department of Life Science and
the Research Institute for Natural Sciences
College of Natural Sciences
Hanyang University
Seoul 133-791, Korea
[2]Department of Biotechnology
Konkuk University
Chungju 380-701, Korea
[3]Samsung Biomedical Research Institute
Department of Molecular Cell Biology
Sungkyunkwan University School of Medicine
Suwon 440-746, Korea
[4]Department of Internal Medicine
College of Medicine Hanyang University
Seoul 133-791, Korea
[5]Corresponding author: Tel, 82-2-2290-0957;
Fax, 82-2-2296-5996; E-mail, cgkim@hanyang.ac.kr
*These two authors contributed equally to the work

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Abbreviations: BAT, brown adipose tissue; GCV, ganciclovir; HSV-TK, herpes simplex virus thymidine kinase; UCP1, uncoupling protein 1; WAT, white adipose tissue

Abstract

Adipose tissue is an important endocrine regulator of glucose metabolism and energy homeostasis. Researches have focused on this tissue not only as a target for pharmacotherapy of obesity and insulin resistance but also as an endocrine tissue with leptin secretion and high insulin sensitivity. Brown adipose tissue (BAT) additionally plays a unique role in thermoregulation through the mitochondrial uncoupling protein 1 (UCP1), which uncouples oxidative phosphorylation. As a genetic tissue ablation model of BAT, we made transgenic mice expressing herpes simplex virus thymidine kinase (HSV-TK) driven by the brown adipocyte-specific UCP1 minimal regulatory element. The HSV-TK transgene was expressed specifically in BAT and more than 35% increase of apoptosis was induced by ganciclovir (GCV) treatment. Nevertheless, the expression level was not high enough to induce BAT ablation in GCV-treated adult mice. Importantly, however, we found that brown adipocytes in the periphery of interscapular BAT were transformed into white adipocyte-like unilocular cells. These cells express white adipocyte-specific leptin protein but are different in the ultrastructure of mitochondria from classical white adipocytes. Our data indicates that atrophy of BAT causes transformation into white adipocyte-like cells in the adult mouse and also suggests that further molecular understanding of adipocyte plasticity using our transgenic mouse model might be beneficial for the development of anti-obesity/anti-diabetic therapies.

Keywords: adipocyte differentiation; brown adipose tissue; energy homeostasis; genetic tissue ablation; transgenic mice

Introduction

Adipose tissue is an important endocrine regulator of glucose metabolism and energy homeostasis. This function is closely linked to obesity, one of the most common metabolic diseases, and a major risk factor for non-insulin-dependent adult-onset *diabetes mellitus*, hypertension, hyperlipidemias, and atherosclerosis. In most mammals, there are two types of adipose tissue, white and brown adipocytes. Both can store energy in the form of triglycerols and express many kinds of common adipocyte-specific genes, such as *aP2* and *PPAR*γ (Moinat et al., 1995; Tai *et al*., 1996; Siegrist-Kaiser *et al*., 1997), and thus brown adipose tissue (BAT) and white adipose tissue (WAT) are largely indistinguishable in terms of their function. However, the mitochondrial protein uncoupling protein1 (UCP1) is expressed exclusively in BAT (Cinti *et al*., 1997; Dessolin *et al*., 1997). Because UCP1 mediates mitochondrial proton leak, decreasing ATP production (Jacobsson *et al*., 1985; Ricquier *et al*., 1991), it has been suggested that BAT uses fatty acids for cold- and diet-induced heat production while WAT provides lipids as metabolic substrates for other tissues (Himms-Hagen, 1990).

Earlier a novel technique was adapted where adipose tissue could be ablated genetically at any specific developmental stage and/or specific physiological condition by exploiting the herpes simplex virus thymidine kinase (HSV-TK) gene as a toxin gene (Ko *et al*., 1998). An advantage using HSV-TK to induce cell death is that thymidine kinase is not toxic by itself, and thus offers a conditional killing mechanism for proliferating cells by treatment with ganciclovir (GCV). Plasmid, pUCP-TK, was constructed which contains HSV-TK gene driven by the 220 bp core enhancer (corresponding to a region from -2,530 to -2,310 bp) and the 98 bp proximal promoter of the murine UCP1 gene (Ko *et al*., 1998; see also Figure 1A). The 220 bp core enhancer was identified as responsible for brown adipocyte-specific and catecholamine-stimulated expression of UCP1 (Cassard-Doulcier *et al*., 1988; Kozak *et al*., 1994; Cao *et al*., 2001). This region also contains a peroxisome proliferator-response element (PPRE) critical for BAT-specific activity and elements contributing to both PKA and p38 MAPK-dependent cAMP responsiveness (Cassard-doulcier *et al*., 1998). UCP1 minimal regulatory element was confirmed to be sufficient to kill brown adipocytes (HIB-1B/pUCP-TK) following the GCV treatment (Ko *et al*., 1998). More than 80% of the differentiating HIB-1B/pUCP-TK cells were dead by the 7th day of treatment with GCV, while undifferentiated cells as

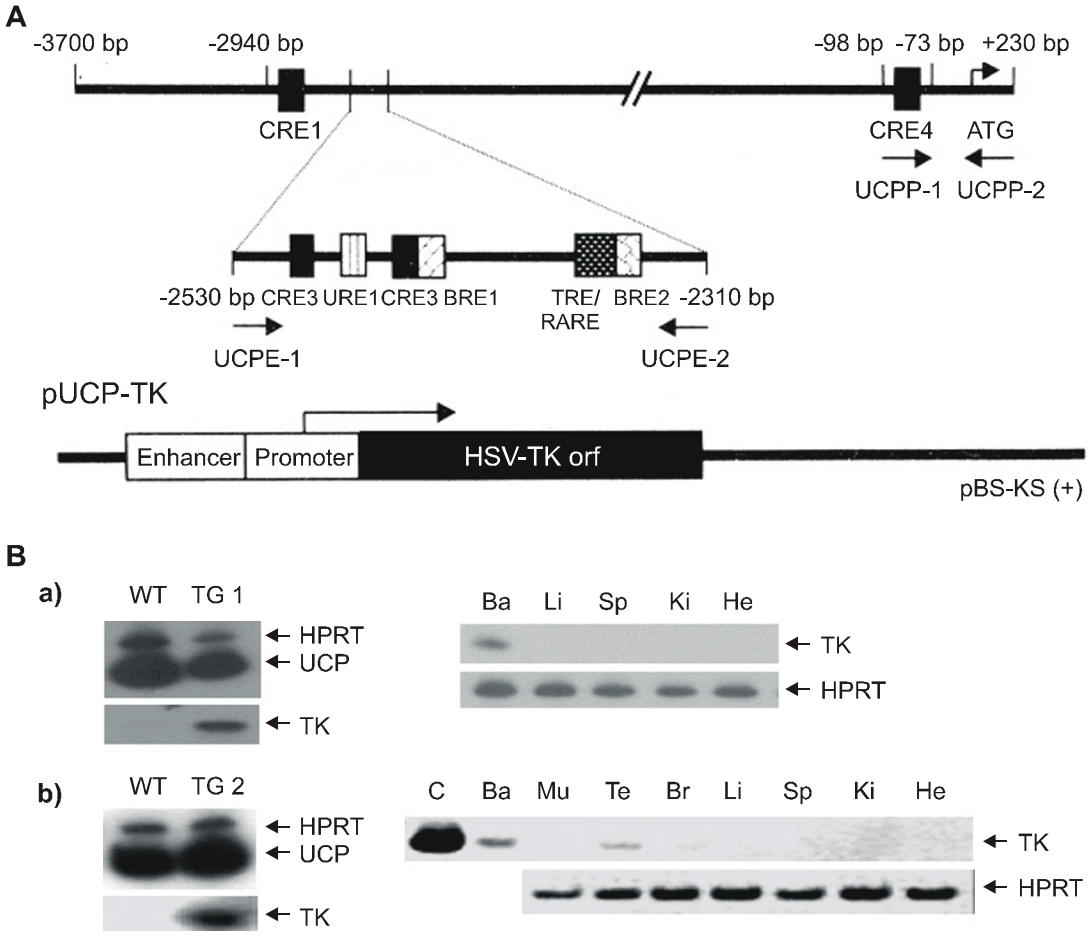

Figure 1. Construction of the UCP-TK transgenic mice. A. Schematic representations of the 5'-flanking region of UCP1 from -3.7 kb to +230 bp and the brown adipocyte specific TK expression vector pUCP-TK are shown. The coordinates are from the data of Ko *et al.* (1998). The 220 bp enhancer region is enlarged, and cis-acting elements are marked with filled boxes. Horizontal arrows below the map indicate positions of oligonucleotide primers used for the PCR cloning of the minimal enhancer sequences and promoter sequences, respectively. A bent arrow indicates a transcription start-site. orf, open reading frame. B. Expression of the TK transgene in the transgenic offspring of two germ line-transmitted female founders (TG1 and TG2). Both comparison of TK expression level in the BAT of UCP-TK mouse to endogenous UCP1 expression (left panel) and expression profile of the UCP-TK transgene in different tissues (right panel) of the a-III-5 transgenic female (a) and b-III-37 transgenic male (b) were shown. HPRT (hypoxanthine phosphoribosyltransferase) served as an internal control and pUCP-TK vector itself (lane C) served as a positive control of PCR reactions. Wild-type littermates (WT) were also served as controls in each test of transgenic offspring (TG). Ba, BAT; Br, brain; He, heart; Ki, kidney; Li, liver; Mu, muscle; Sp, spleen; Te, testis.

well as the cells induced by the default differentiation condition by omitting some inducing factors were not killed by GCV. In this study, transgenic mouse lines exhibiting germ-line transmission of pUCP-TK were made to analyze BAT function in adult mice following GCV administration.

Materials and Methods

Animal care and generation of transgenic mice

All experiments followed the guidelines of the Experimental Animal Usage and Care of the Korean Food and Drug Administration. Animals were housed in plastic shoebox cages with wire lids and wood shaving bedding in an air-conditioned room. They were provided with food and water *ad libitum* and a 12 h light/dark cycle was used. The offsprings were weaned about three weeks after birth, digit-marked, and separated by gender. To generate transgenic mice lines, the pUCP-TK expression vector (Figure 1A; Ko *et al*., 1998) was linearized with *Xho*I and *Not*I and microinjected into pronuclear-stage eggs prepared from the FVB/N mice. Microinjected zygotes were implanted into the uteri of pseudo-pregnant surrogate mothers. Transgenic mice with germ-line transmission were acquired by breeding F_0 mice with wild-type FVB/N mice. Genomic PCR of DNA prepared either from a tail fragment or peripheral blood was used to genotype the mice. Five ng of DNA was subjected to PCR of 35 amplification cycles of 94°C 1 min, 60°C 30 sec, and 72°C 2.5 min in a solution containing UCPP-1 and TK-2 primers (see Figure 1A). HPRT was used as an endogenous control. PCR primer sequences were as follows: UCPP-1 (the UCP promoter from -98 to -75 nt), 5'-CAT GCA GCT CTT TGG AGA CCT GGG-3'; TK-2 (the HSV-TK gene from +271 to +290 nt), 5'-GCC ACC AAG ATC TGC GGC AC-3; HPRT 1R, 5'-CAC AGG ACT AGA ACA CCT GC-3'; HPRT 2R, 5'-GCT GGT GAA AAG GAC CTC T-3'.

Ganciclovir injection and weight measurement

GCV injection was performed according to the procedure described by Markkular *et al*. (1996). Two hundred μg of GCV per 20 g of body weight was injected intra-peritoneally twice a day for two weeks and once a day afterwards. Weight measurement was performed once a day. The cold-exposed group was acclimated for 12 h a day at 4°C and the standard GCV injection procedure was followed.

Tissue specimen, immunohistochemical staining and image analysis

Tissue samples were prepared from humanely killed mice by cervical dislocation and each sample was fixed in 4% paraformaldehyde within 10 min of sacrifice. Tissue slices were prepared by the standard paraffin section method. After examining the specimen morphology by H/E staining, appropriate adjacent serial sections were used for further analysis. Immunohistochemistry was performed basically following Cancello's procedures (Cancello *et al*., 1998) using rabbit polyclonal antibody against mouse leptin (Santa Cruz) and goat polyclonal antibody against mouse UCP1 (Santa Cruz). Dewaxed sections were subjected to endogenous peroxidase blocking with 0.3% H_2O_2 in

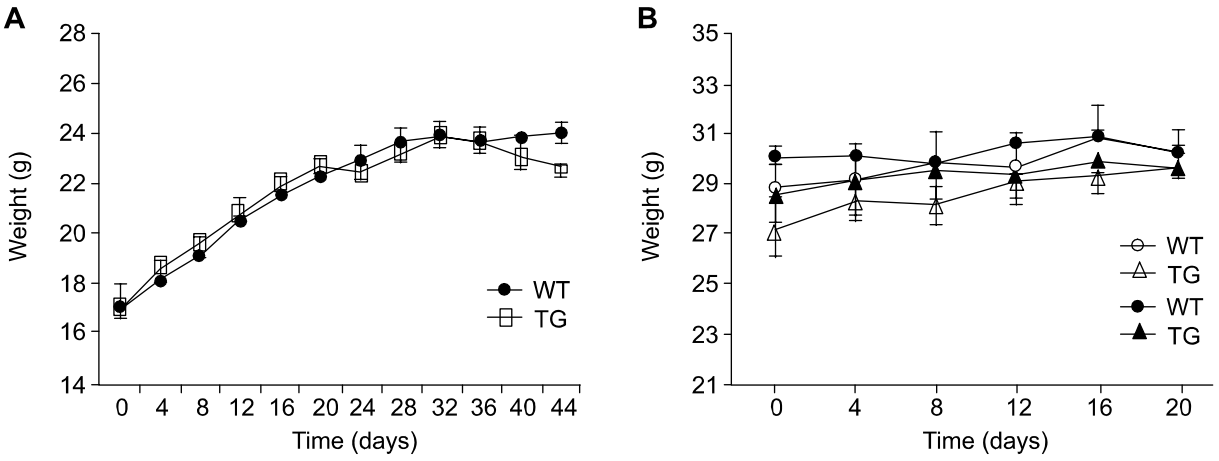

Figure 2. Obesity was not observed on the GCV-treated transgenic mice. Wild type and UCP-TK mice were injected with GCV or PBS (Mock) for more than 3 weeks. Body weight changes of 5 week-old mice (A) and cold acclimated 14 week-old mice (B) were measured every day after GCV injection and the data is represented at 4-day intervals showing mean±SD. Two 5 week-old mice and three cold-acclimated 14 week-old mice were employed and the experiments repeated at least twice. WT, wild type littermate; TG, UCP-TK transgenic mice; +/-, performance of cold acclimation at 4°C for 12 h per day (+) or not (-) during GCV treatment.

methanol for 30 min and then incubated in PBS containing 3% horse serum, 0.1% Triton X-100 for 2 h (Kim *et al*., 2001). After incubation with each primary antibody (1:200 in 2% horse serum and 0.1% Triton X-100 in PBS) at 4°C overnight, a biotinylated anti-goat antibody (Vector, 1:200 for UCP1) or a peroxidase-linked anti-rabbit antibody (ECL, 1:400 for leptin) was applied for 2 h. Colorimetric visualization was achieved by treatment of specimens with 0.5 mg/ml DAB and 0.1% H_2O_2 in PBS for 1-10 min. Quantification of microscopic image was made using Image-Pro Plus program (Media Cybernetics).

Transmission electron microscopy

Minute interscapular BAT specimens (less than 1 mm^2) were fixed with modified Karnovsky fixative containing 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 at 4°C for 2 h (Karnovsky, 1965; Ali *et al*., 1988). Specimens were postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer on ice for 1 h, dehydrated in ethanol, and embedded in Epon 812. Ultra-thin sections were made using an ultramicrotome (Reichert-Jung) equipped with a diamond knife (Diatome), and routinely stained with uranyl acetate followed by lead citrate (Reynolds, 1963). Stained sections were examined using an electron microscope (Hitachi 600) at

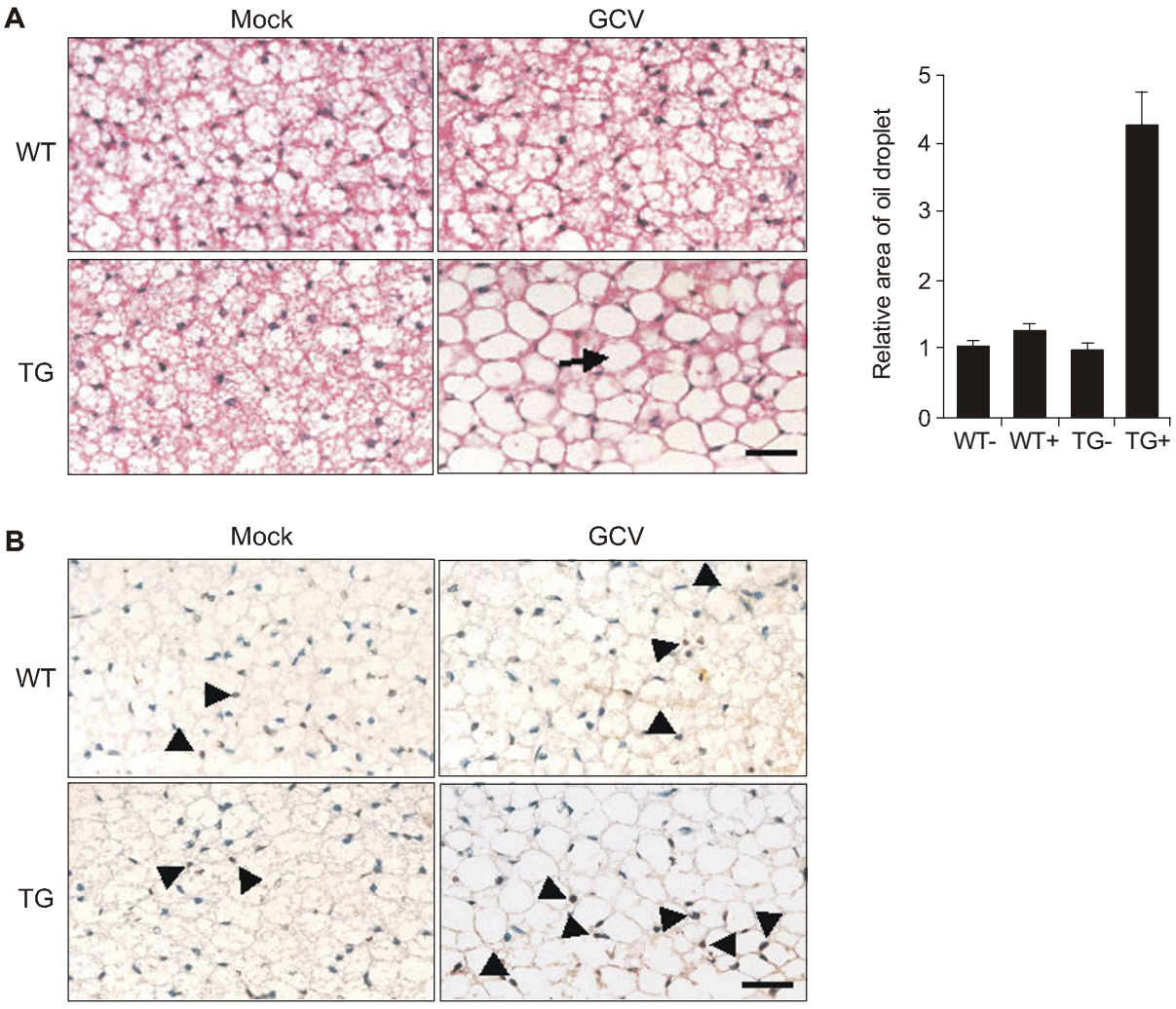

Figure 3. BAT of GCV-treated UCP-TK mice was transformed to WAT-like cells in the periphery with mild increase of apoptosis. A. Paraffin-sectioned interscapular BAT specimens (transgenic offspring and their wild type littermate with or without treatment of GCV) were prepared and stained with hematoxylin/eosin. The peripheral regions of BAT are shown. Peripheral unilocular adipocytes in BAT of the GCV-treated UCP-TK mouse are readily visible (arrow). Relative oil droplet size was calculated after measuring more than 100 cross- sectioned areas of oil droplets for each sample using Image-Pro Plus program (Media Cybernetics). B. TUNEL assays were performed on BAT sections. The peripheral regions of BAT are shown. Apoptotic nuclei were brown (arrow), whereas non-apoptotic nuclei counterstained with hematoxylin were dark blue. bar, 25 um.

maximal 75-kV accelerating voltage.

RT-PCR and *In situ* RT-PCR

Total RNA extracted from tissues using RNAzol™B (TEL-TEST) was subjected to reverse transcription reaction and RT-PCR (Kim, 1996; Park *et al.*, 2001). The sequences of the primers were as follows: TK-3 (the HSV-TK gene from +124 to +143 nt), 5'-TGC CAT CAA CAC GCG TCT GC-3'; TK-4 (the HSV-TK gene from +325 to +344 nt), 5'-ACG ATA TCG TCG CGC GAA CC-3'; UCP-1 (the mouse UCP1 gene from +411 to +430 nt), 5'-GAC CAT GAC CAC CCT GGC AA-3'; UCP-2 (the mouse UCP1 gene from +601 to +618 nt), 5'-GAA CAC TGC CAC ACC TCC AG-3'. RT-PCR products were sequenced to confirm that they originated from the transgene.

Terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) staining

A TUNEL assay for the detection of DNA fragmentation by apoptosis was carried out using the *In Situ* Cell Death Detection Kit (Roche Applied Science) according to the manufacturer's instructions. Non-apoptotic nuclei were distinguished by counterstaining with Harris' hematoxylin. Apoptotic cells were quantified by counting the number of apoptotic nuclei under the microscope at 400 X magnification in more than 10 random fields per sample.

Results and Discussion

Production of transgenic mice with BAT-specific TK gene expression

Among three UCP-TK founder mice, two female mice exhibited germ-line transmission of the transgene whereas a male founder was sterile, consistent with previous reports that transgenic mice harboring the HSV-TK gene produce abnormal sperms due to a testis specific extra transcription initiation site within the HSV-TK gene (Robert *et al.*, 1990; Salomon *et al.*, 1995; see also Figure 1B). The copy number of the transgene was estimated to be approximately 5 by genomic Southern blotting using UCP1 and HSV-TK gene probes and genomic DNA prepared from various tissues from transgenic mice (data not shown). The overall transmission rate of the UCP-TK transgene was approximately 50% from the third generation (n = 51) and no gross anatomical or histological abnormalities was observed (data not shown), which suggests that the insertion of our transgene *per se* does not cause any apparent phenotypic effect. UCP-TK was expressed specifically in BAT of UCP-TK mice when RT-PCR was performed with RNA extracted from various tissues of transgenic mice, although mild ectopic expression was also detected in testes of male offspring (Figure 1B). The expression level of transgene, however, was less than one tenth of the endogenous UCP1 gene in F3 offspring of both transgenic lines (Figure 1B).

Obesity was not induced by GCV treatment of adult transgenic mice

In spite of low BAT-specific expression of the transgene, whether GCV treatment had any effect on the obesity was examined. If BAT thermogenesis were actively involved in obesity and GCV administration to adult transgenic UCP-TK mice led to BAT ablation, obesity might be predicted. GCV was injected into 5-week-old UCP-TK mice for 6 weeks and their body weight was measured once a day. As seen in Figure 2A, there was no significant change in body weight compared to mock-treated wild type controls. Since UCP1 gene expression is activated further by cold acclimation (Jacobson *et al.*, 1985), we repeated this experiment in cold acclimated mice and found no significant weight change in the cold exposed group (data not shown). These results were reproducible even when a similar experiment was performed on 14 week-old mice (Figure 2B).

GCV treatment of adult transgenic mice promotes transformation of brown adipocytes into white adipocyte-like cells in the periphery of scapular BAT

To understand possible relationship of obesity in the GCV-treated transgenic mice, the BAT of the GCV-treated transgenic mice was examined. Curiously,

Table 1. Quantification of apoptotic cells. The number of cells was scored under the microscope after performing TUNEL assays of BAT tissues from GCV-treated wild type and UCP-TK transgenic mice. Normal wild-type and GCV-untreated transgenic mice were also included as controls for monitoring potential GCV side effects.

	Negative cell numbers	Positive cell numbers	Total cell numbers	Positive cells (%)	Relative apoptotic index (fold)
WT-	457	66	523	13	0.93
WT+	432	57	489	12	0.86
TG-	408	69	477	14	1.00
TG+cent	298	69	365	19	1.36
TG+peri	251	89	340	26	1.86

WT, wild-type mouse; TG, transgenic mouse; -, no GCV injected; +, GCV injected; cent, central region of BAT; peri, peripheral region of BAT.

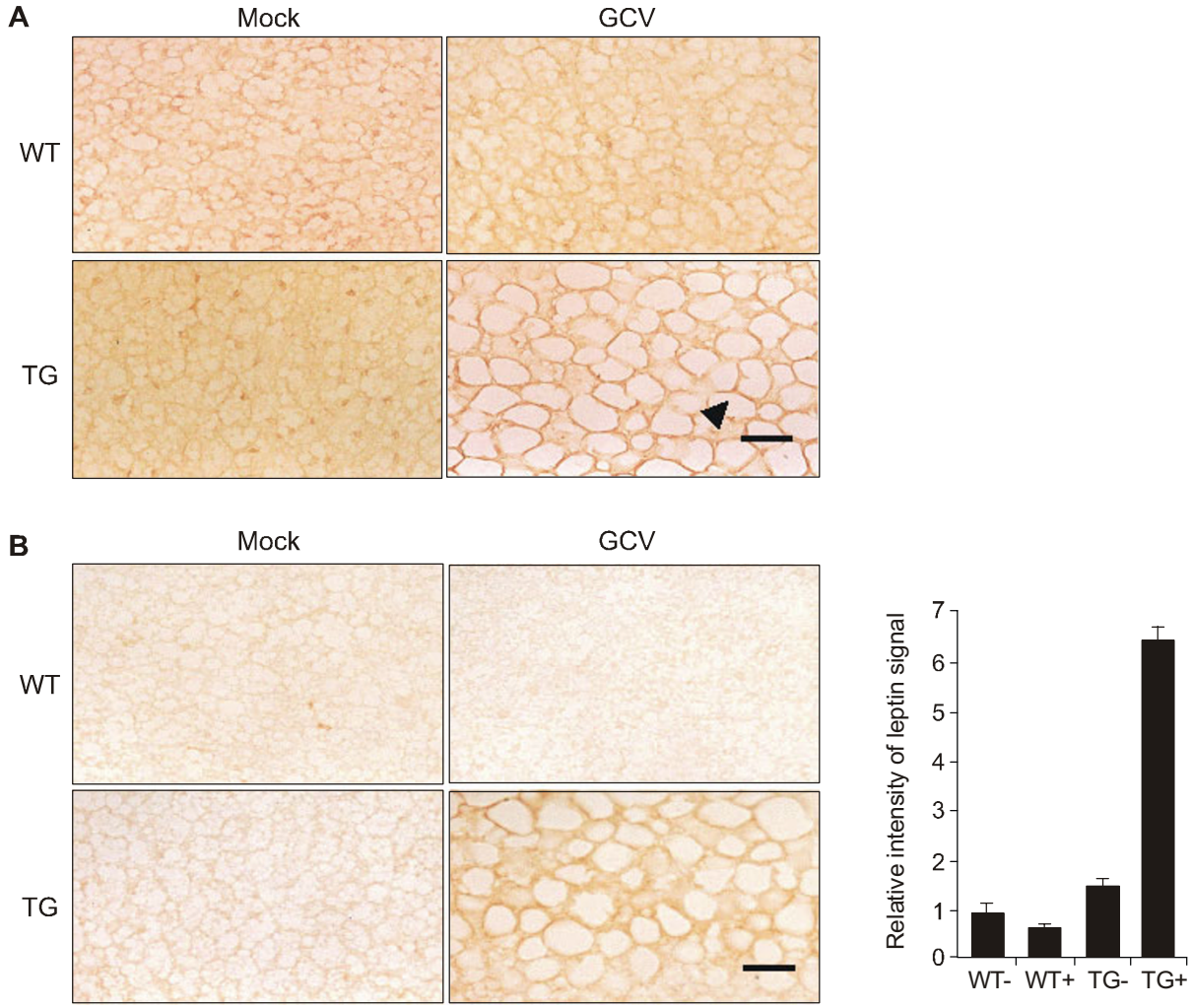

Figure 4. WAT-like cells at the periphery of BAT in GCV-treated UCP-TK mice. Immunohistochemical localization of UCP1 (A) and leptin (B) is shown in the peripheral BAT regions of PBS- (mock) or GCV-treated wild type and UCP-TK mice. Both inner multilocular and peripheral unilocular adipocytes were UCP-positive in all groups, whereas only unilocular adipocytes in BAT of the GCV-treated UCP-TK mouse were leptin-positive. Relative intensity of leptin signal in the peripheral BAT was calculated after measuring the intensity of signal in more than 100 brown adipocytes for each sample using Image-Pro Plus program (Media Cybernetics). bar, 25 μm.

BAT mass was still existed in the interscapular region. At light microscopy level, brown adipocytes have cytoplasmic lipids arranged as numerous small droplets (multilocularity), while white adipocytes have cytoplasmic lipids arranged in a unique vacuole (unilocularity) (Cinti, 2002). Importantly, unilocular cells were seen at the periphery of BAT in GCV-treated UCP-TK mice but not in GCV-treated wild type littermates or mock-treated wild type and transgenic mice (Figure 3A). They constituted up to 50% of the cell population of the scapular BAT and were similar to WAT in morphology containing more than 4-fold larger lipid droplet masses of the control groups (Figure 3A).

To examine whether the appearance of unilocular cells at the periphery of BAT in GCV-treated UCP-TK mice is related to GCV-induced apoptotic cell death, a TUNEL assay was performed on BAT sections. Apoptotic cell numbers in the central and peripheral portions of BAT in GCV-treated UCP-TK mice were increased 36% and 86%, respectively, compared to GCV-untreated transgenic mice (Table 1; see also Figure 3B). Although the appearance of a peripheral unilocular cell mass is positively correlated with an increase in apoptotic cells, not all of the unilocular cells were undergoing apoptosis.

The existence of BAT in GCV-treated transgenic mice suggests that either low expression of HSV-TK in the adult BAT is not sufficient to induce GCV-mediated BAT ablation or that moderate ablation of BAT due to the slow proliferation of brown adipocytes in adult BAT does not significantly alter body weight. Since cold exposure of the 6-month-old rats for 5

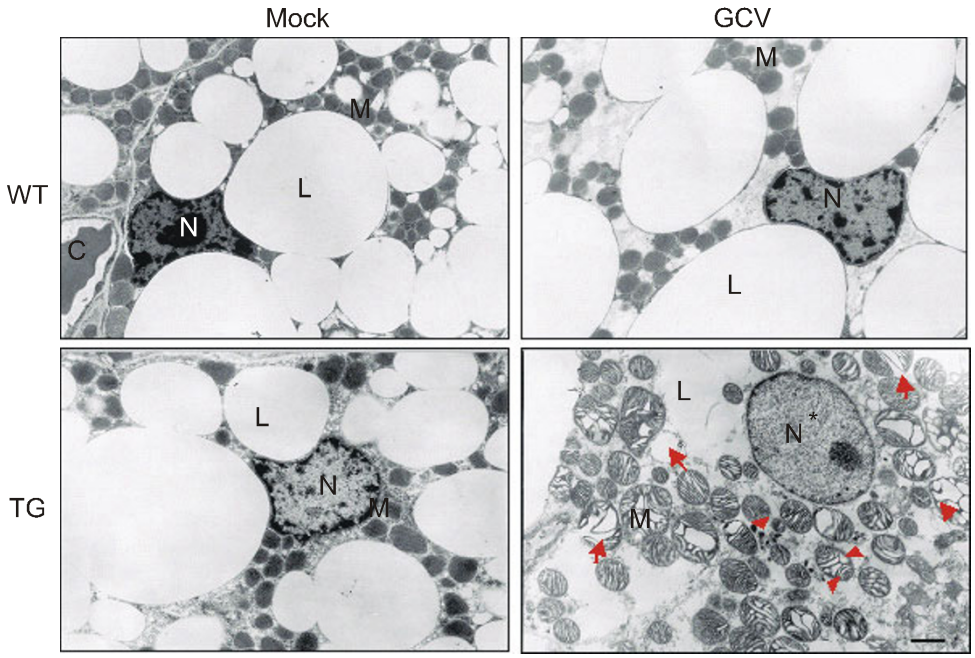

Figure 5. Ultrastructures of interscapular adipocytes of PBS- (mock) or GCV-treated wild type and UCP-TK mice. An arrow and arrowheads mark degenerating and degrading mitochondria, respectively. An asterisk also marks the nucleus with reduced heterochromatin. N, nuclei; M, mitochondrion; L, lipid droplet. Viewing magnification, ×8,000.

days produced an additional increase of 29% in mature brown adipocyte cell size (Florez-Duquet *et al*., 1998), brown adipocyte proliferation/differentiation-dependent GCV activity is not likely a cause. Thus, GCV treatment of adult UCP-TK mice was not sufficient to induce massive apoptosis of cells required to cause BAT ablation, but rather induced transformation of brown adipocytes into white adipocyte-like cells on the periphery of BAT.

White adipocyte-like cells express leptin but differ from classical white adipocytes in ultrastructure

To confirm whether the peripheral unilocular cell-mass is derived from brown adipocytes and indicative of white adipocytes, immunohistochemical localization of UCP1 and leptin proteins, well-known markers of brown and white adipocytes, respectively, were undertaken. Although BAT is also able to produce leptin at birth, the classic multilocular UCP-expressing interscapular brown adipocytes do not produce immunohistochemically detectable levels of this protein in lean mice (Cancello *et al*., 1998). Indeed, whereas UCP1 was expressed in entire BAT area and at a slightly higher level in the unilocular cell-mass (Figure 4A), leptin was expressed only in the peripheral unilocular cell-mass (Figure 4B). Thus, this unilocular cell-mass is distinguished from the peripheral unilocular adipocytes, usually considered as "contaminant", which shows UCP1-negative and leptin-positive (Cancello *et al*., 1998). The relative intensity of leptin signal in the peripheral unilocular cell-mass was more than 6-fold higher to those of other control groups.

Furthermore, when the subcellular organization of the white adipocyte-like unilocular cells was examined by electron microscopy, the overall cellular structure was similar to that typically seen in white adipocytes, which are filled with large lipid droplets (Figure 5; Kubo *et al*., 1998). Unlike white adipocytes, however, the nucleus exhibited the typical spherical shape of BAT and the heterochromatin region was reduced significantly without a change in the size of nucleus. Nuclei seen in these cells were also different from those seen in apoptotic cells, which exhibit uneven aggregation of heterochromatin with nuclear fragmentation, or from those seen in normal cells, which contain large amounts of heterochromatin near the nuclear membrane (Figure 5; Kubo *et al*., 1998). Mitochondria showed several characteristics of white adipocytes, such as heterogeneity in size and a significantly reduced number of cristae; moreover, partially degenerating mitochondria were also observed. Similar degenerating brown adipocytes were also reported in BAT of UCP1 or NE/E knockout mice (Enerback *et al*., 1997; Thomas and Palmiter, 1997), as well as in BAT of obese mice, fasted mice or mice bred at high temperature (Hull and Vinter, 1984; Muralidhara and Desautels, 1994; Frederich *et al*., 1995; Cinti *et al*., 1997; Cancello *et al*., 1998), implying that the

onset of brown adipocyte degeneration may be related to decreases in thermogenic activity although the detailed mechanism of obesity-linked BAT atrophy is not yet fully understood.

Perspective

Our overall data strongly suggests that unilocular cells in the peripheral BAT of transgenic mice are in a transition state from brown- to white-adipocytes. Indeed, recent data have stressed the plasticity of the adipose organ in adult animals, suggesting that, under peculiar conditions, fully differentiated white adipocytes can transdifferentiate into brown adipocytes, and vice versa (see review Cinti, 2002). Multilocular, mitochondria-rich adipocytes appeard in WAT of rats treated with the β3-adrenoceptor agonist (Himms-Hagen *et al*., 2000), while β3 adrenoceptor knockout in mice depresses the occurrence of brown adipocytes in white fat. Recently, Tiraby *et al*. (2003) also demonstrated that typical features of brown fat cells, including expression of UCP1 mRNA, were acquired *in vivo* by injection of the PGC-1α adenovirus in mouse white fat. Thus, the ability of the adipose organ to interconvert its main cytotypes in order to meet changing metabolic needs is highly pertinent to the physiopathology of obesity and related to therapeutic strategies. Further molecular understanding of adipocyte plasticity might be beneficial for the development of anti-obesity and/or anti-diabetic therapy as well as for basic scientific understanding of adipocytes.

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