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The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation

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Barbatelli G, Murano I, Madsen L, Hao Q, Jimenez M, Kristiansen K, Giacobino JP, De Matteis R, Cinti S. The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. *Am J Physiol Endocrinol Metab* 298: E1244–E1253, 2010. First published March 30, 2010; doi:10.1152/ajpendo.00600.2009.—The origin of brown adipocytes arising in white adipose tissue (WAT) after cold acclimatization is unclear. Here, we demonstrate that several UCP1-immunoreactive brown adipocytes occurring in WAT after cold acclimatization have a mixed morphology (paucilocular adipocytes). These cells also had a mixed mitochondrioma with classic "brown" and "white" mitochondria, suggesting intermediate steps in the process of direct transformation of white into brown adipocytes (transdifferentiation). Quantitative electron microscopy disclosed that cold exposure (6°C for 10 days) did not induce an increase in WAT preadipocytes. β_3-adrenoceptor-knockout mice had a blunted brown adipocyte occurrence upon cold acclimatization. Administration of the β_3-adrenoceptor agonist CL316,243 induced the occurrence of brown adipocytes, with the typical morphological features found after cold acclimatization. In contrast, administration of the β_1-adrenoceptor agonist xamoterol increased only the number of preadipocytes. These findings indicate that transdifferentiation depends on β_3-adrenoceptor activation, whereas preadipocyte recruitment is mediated by β_1-adrenoceptor. RT-qPCR experiments disclosed that cold exposure induced enhanced expression of the thermogenic genes and of genes expressed selectively in brown adipose tissue (iBAT) and in both interscapular BAT and WAT. β_3-adrenoceptor suppression blunted their expression only in WAT. Furthermore, cold acclimatization induced an increased WAT expression of the gene coding for C/EBPα (an antimitotic protein), whereas Ccna1 expression (related to cell proliferation) was unchanged. Overall, our data strongly suggest that the cold-induced emergence of brown adipocytes in WAT predominantly reflects β_3-adrenoceptor-mediated transdifferentiation.

development; white adipose tissue; interscapular brown adipose tissue

ALL MAMMALS ARE PROVIDED WITH TWO DIFFERENT TYPES of adipose tissues: white adipose tissue (WAT), made up of unilocular (UL) white adipocytes, and brown adipose tissue (BAT), made up of multilocular (ML) brown adipocytes. WAT stores chemical energy (e.g., triglycerides), whereas BAT dissipates it in the form of heat (thermogenesis) (10, 13, 14). It is widely accepted that WAT and BAT are fat depots located in different anatomic sites. However, recent studies have shown that, at least in some murine strains, most of the fat depots are made up of both tissues (for a recent review, see Ref. 18).

It is well established that β-adrenergic stimulation, such as cold acclimatization, not only activates the brown adipocytes in interscapular BAT but also induces the appearance of novel brown adipocytes in WAT (2, 15, 16, 25, 35). However, the origin of these brown adipocytes occurring in WAT is still unclear. Recent data suggest that interscapular brown adipocytes derive from a common precursor that is also able to differentiate into skeletal muscle (3, 57, 63). This common precursor expresses the early marker of myogenesis Myf5 that was shown, by in vivo mapping studies, to be absent from the lineage of the brown adipocytes appearing in WAT upon adrenergic stimulation (57, 63), suggesting that they could have a different origin. Data from our and other laboratories suggest that most of the newly formed brown adipocytes arising upon β-adrenoceptor agonist stimulation derive from the direct transformation of mature white adipocytes (34, 37). This is in line with previous work showing that, in murine WAT, cold acclimatization induces neither an increase in DNA content nor an increased adipocyte number (24, 30, 46, 48). Gene expression studies combined with morphometry and immunohistochemistry have demonstrated that the emergence of brown adipocyte in WAT after 10 days of cold exposure is blunted in β_3-adrenoceptor-knockout (β_3-KO) compared with wild-type (WT) mice (40), suggesting a crucial role for β_3-adrenoceptor in the appearance of novel brown adipocytes in this tissue.

The white/brown plasticity of adipose tissues might have considerable medical implications, since the brown-like phenotype seems to correlate with a reduced propensity for developing obesity and diabetes in mice (1, 4, 42, 43, 48). It also appears to be involved in the ability of the β_3-adrenoceptor agonist CL316,243 to curb obesity induced by a high-fat diet in rodents (22, 32, 33, 35). Therefore, the phenomenon of appearance of brown adipocytes in WAT after cold exposure and acclimatization warrants further research, also taking into account recent evidence for functional brown adipocytes in adult humans (27, 56, 67, 68, 70).

In this study we investigated the fine morphology, gene expression, and role of β_3-adrenoceptor in the cold-induced emergence of brown adipocytes in mouse WAT. Our findings support the notion that the main phenomenon underpinning the appearance of brown adipocytes in WAT is the direct transformation of mature white adipocytes into brown adipocytes.

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The process appears to be mediated by β_3-adrenoceptor, whose action is blunted in β_3-KO mice. However, recruitment of preadipocytes, which seems to be a minor contributor to the phenomenon, does not require a functioning β_3-adrenoceptor and seems to be mediated by β_1-adrenoceptor stimulation.

MATERIALS AND METHODS

β_3-KO mice. C57BL/6J (B6) and 129Sv mice were obtained from BRL (Fullinsdorf, Switzerland). Targeted β_3-adrenoceptor disruption was initially generated on a mixed 129Sv × B6 background. The β_3-KO mice were then backcrossed with B6 and 129Sv mice to obtain mutated mice with the respective purified genetic backgrounds (98.4% homogeneity). The mice used in this study were the offspring of WT and β_3-KO founders from 129Sv and B6 purified genetic backgrounds. Genotyping was performed by Southern blots, as described previously (54). Groups of four 3-mo-old female WT and β_3-KO mice of each genetic background were kept above room temperature (24 ± 1°C; controls) or at 6°C for 1, 2, 3, or 10 days. Animals were housed individually with a 12:12-h light-dark cycle and free access to pellet food and water. Care and handling were in accordance with institutional guidelines. Our experiments were approved by the Ethics Committee for Animal Experiments at the University of Ancona (11 Gen. 2007; protocol no. 585).

Cold acclimatization experiments. Groups of four 10-wk-old female 129Sv mice (Charles River, Milan, Italy) were housed separately and kept above room temperature (24 ± 1°C; controls) or at 6°C for 1, 3, 5, or 10 days.

β_1- and β_3-adrenoceptor agonist administration experiments. Six groups (3 animals each) of 10-wk-old female 129Sv mice (Charles River) kept above room temperature (24 ± 1°C) were given a daily intraperitoneal (ip) injection for 3 or 5 days. Two groups received the β_1-adrenoceptor agonist xamoterol hemifumarate (0.144 mg/kg), two groups received the β_3-adrenoceptor agonist CL316,243 (0.1 mg/kg; both from Tocris Cookson, Bristol, UK), and two groups received saline (100 μl). Mice were euthanized with an ip overdose of ketamine (100 mg/kg, Ketavet 100; Intervet, Milan, Italy) in combination with xylazine (10 mg/kg, Rompum; Bayer, Milan, Italy). For morphology experiments, the mice were immediately perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 5 min.

Real-time quantitative PCR. Real-time quantitative PCR (RT-qPCR) was performed on 129Sv mice. Total RNA from interscapular BAT (iBAT), parametrial WAT, which was selected to represent visceral fat (visceral WAT), and inguinal WAT, taken to represent subcutaneous fat (subcutaneous WAT), was purified with Trizol. cDNA was synthesized and analyzed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), as described previously (44). Primers for RT-qPCR (Table 1) were designed using Primer Express 2.0 (Applied Biosystems).

Light microscopy. iBAT, visceral WAT, and subcutaneous WAT were dissected under a Zeiss OPI1 surgical microscope (Carl Zeiss, Oberkochen, Germany), fixed by immersion (overnight, 4°C), dehydrated, cleared, and finally embedded in paraffin blocks. The tissue was cut into serial 3-μm-thick sections; the first was stained with hematoxylin-eosin for morphological investigations, and the others were processed for immunohistochemistry (see below).

To seek signs of apoptosis (41), we examined 10 random high-power fields (×100) per depot of each mouse as well as all of the sections used for morphometry (×40).

Immunohistochemistry. Immunohistochemistry studies were performed on β_3-KO subcutaneous WAT, visceral WAT, and iBAT and on subcutaneous WAT from cold-exposed mice (which was also used in the experiments with β_1- and β_3-adrenoceptor agonists). The tissues were processed with a polyclonal anti-rat uncoupling protein (UCP)1 antibody raised in sheep (kindly provided by D. Ricquier, Paris, France) according to the avidin-biotin-peroxidase complex method (38). For negative controls, the primary antibody was substituted with sheep IgG. No cross-reaction with UCP2 and UCP3 was observed in tissues expressing high levels of UCP2 (liver) or UCP3 (skeletal muscle).

Morphometry. Morphometry studies were performed on β_3-KO subcutaneous WAT and visceral WAT and on subcutaneous WAT from cold-exposed mice (which was also used in the experiments with β_1- and β_3-adrenoceptor agonists). Adipocytes were identified as UL (containing a single large vacuole), PL (paucilocular; exhibiting a large vacuole surrounded by at least 5 small lipid droplets), or ML (containing more than 5 small, homogeneous lipid droplets). UL, PL, and ML adipocytes were counted, and the proportion of UCP1-immunoreactive (ir) PL and ML cells was calculated in sections by immunohistochemical localization of UCP1 protein. UL adipocyte area was measured on hematoxylin-eosin sections, assuming a spherical shape of these cells. In brief, 300 random adipocyte profiles from different, well-preserved areas of each depot were drawn using a digital image analysis system, and their surface area was measured by light microscopy (×20) using the Nikon Lucia Image program (Laboratory Imaging, Praha, Czech Republic).

Electron microscopy. Small fragments of tissue (subcutaneous WAT from 129Sv β_3-KO mice, iBAT from cold-exposed mice, and subcutaneous WAT for the β_1- and β_3-adrenoceptor agonist experiments) were used for electron microscopy (EM) studies (47).

Sections from the subcutaneous depot were from areas containing ML cells. In this study, we considered as preadipocytes all poorly differentiated cells surrounded by a distinct basal membrane located

Table 1. *Primers for RT-qPCR*

Gene	Forward Primer	Reverse Primer
C/EBPα	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
C/EBPβ	GACACGGGACTACGCAACAC	AACCCCGCAGGAACATCTTTA
Cidea	TGCTCTTCTGTATCGCCCAGT	GCCGTGTTAAGGAATCTGCTG
Cox8b	GAACCATGAAGCCAACGACT	GCGAAGTTCACAGTGGTTCC
Eva1	GTCCCAACCAGACCATCAAC	CTCCATCTTGCTCTGGAAGC
PGC-1α	CATTTGATGCACTGACAGATGGA	CCGTCAGGCATGGAGGAA
PRDM16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
PsatI	TACCGCCTTGTCAAGAAACC	AGTGGAGCGCCAGAATAGAA
Serpin3ak	GGC TGAAGGCAAAGTCAGTGT	TGGAATCTGTCCTGCTGTCCT
UCP1	AGCCGGCTTAATGACTGGAG	TCTGTAGGCTGCCCAATGAAC
Ccna1	GCTGGCCATGAACTACCTG	AGAGGCCACGAACATGC

RT-qPCR, real-time quantitative PCR; C/EBPα and -β, CCAAT/enhancer binding protein-α and -β; Cidea, cell death-inducing DNA fragmentation factor, α-subunit-like effector A; Cox8b, cytochrome *c* oxidase, subunit VIIIb; Eva1, epithelial V-like antigen 1; PGC-1α peroxisome proliferator-activated receptor-γ coactivator-1α; PRDM16, PR domain containing 16; Psat1, phosphoserine aminotransferase; Serpin3ak, serine or cysteine peptidase inhibitor, clade A, member 3K; UCP1, uncoupling protein-1; Ccna1, cyclin A1.

in the capillary wall in a pericytic position, as described in our studies and in articles by other researchers (5, 19, 45, 50, 59, 61). Preadipocytes were counted in each section and compared with the total number of adipocytes. Results are given as preadipocyte density (preadipocyte number/100 adipocytes).

Statistical analysis. Results are given as means ± SE. Differences between group means were analyzed by two-way ANOVA (InStat; GraphPad, San Diego, CA). Differences between groups were considered significant when $P \leq 0.05$.

RESULTS

Brown adipocytes in WAT have two distinct morphologies. The majority (>95%) of adipocytes found in the visceral and the subcutaneous depots of control mice (24°C) had the classic features of white adipocytes (UL cells devoid of UCP1 immunoreactivity).

Brown adipocytes (UCP-ir cells) were seen only in the subcutaneous depot in 129Sv mice (Fig. 1). They displayed two distinct morphologies, which characterized them as PL or as classic (ML) adipocytes; the former cells (43% of all brown adipocytes) were larger and had a peripheral nucleus, a central large lipid droplet and several small lipid droplets in the periphery of the cytoplasm (Fig. 2, *A* and *B*), whereas ML cells (57% of all brown adipocytes) had the typical morphology of brown adipocytes, i.e., rounded with a central nucleus and several small uniform lipid droplets in the cytoplasm (Fig. 2, *E* and *F*).

On EM, numerous mitochondria were detected in both PL and classic ML brown adipocytes. Those found in ML cells displayed the typical features of classic "uncoupled brown fat", i.e., they were large with numerous transverse cristae (13–15, 17) (Fig. 2, *G* and *H*), whereas those of PL cells exhibited a mixture of classic brown adipocyte mitochondria ("brown" mitochondria) and of elongated mitochondria similar to those found in white adipocytes ("white" mitochondria). Mitochondria with an intermediate morphology were also detected in PL cells (Fig. 2 *C* and *D*). Interestingly, white-like as well as intermediate mitochondria were also seen in some classic brown adipocytes (Fig. 2*H*).

Thus PL cells, albeit ML and UCP1-ir, i.e., brown, displayed intermediate features between brown and white adipocytes. Some UCP1-negative PL adipocytes were also observed. Thus transitional steps from white UL adipocytes to brown ML adipocytes could be those shown in Fig. 3.

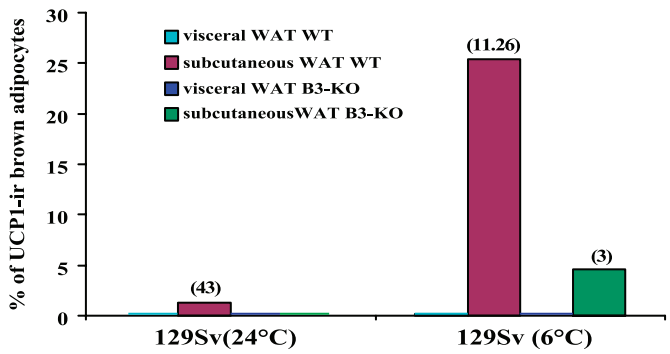

Fig. 1. Quantification of brown adipocytes in white adipose tissue (WAT) pads. Proportion of uncoupling protein-1-immunoreactive (UCP1-ir) adipocytes in visceral and subcutaneous WAT of 129Sv wild-type (WT) and β_3-adrenoceptor knockout (β_3-KO) mice. Animals were kept at 24 or 6°C for 10 days. Numbers in parentheses above bars indicate the proportion of UCP1-ir brown adipocytes with paucilocular (PL) features (see text and Fig. 2) in that depot. Means ± SE.

Cold-acclimatized brown adipocytes arise in two different "white" fat depots. After 10 days' exposure to 6°C, brown adipocytes increased by 20 times ($P < 0.01$) in subcutaneous WAT; they also increased in visceral WAT, but the difference here was not significant (Fig. 1 and Supplemental Table S1; Supplemental Material for this article can be found on the *AJP-Endocrinology and Metabolism* web site). Again, both classic and PL forms of brown adipocytes were detected (Fig. 2). High-power light microscopy can often recognize apoptosis with confidence (41). However, no signs of apoptosis or macrophage infiltration were detected in the tissues of any WAT depot in cold-acclimatized mice. Furthermore, upregulation of expression of genes involved in apoptotic pathways such as *Bax*, *Bcl2l14*, *Bok*, *p53*, and *Tnfsf10* was not observed (not shown).

Gene expression studies showed that cold acclimatization led to a rapid induction of the key thermogenic genes UCP1, PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1α), and C/EBPβ (CCAAT/enhancer binding protein-β) (52, 53, 55) in the three depots (iBAT, visceral WAT, and subcutaneous WAT) (Fig. 4*A*). PRDM16 (PR domain containing 16), Eva1 (epithelial V-like antigen 1), and Cox8b (cytochrome *c* oxidase, subunit VIIIb) are preferentially expressed in iBAT (iBAT marker genes) (58). Their expression was induced in all three depots, and, as expected, their mRNA levels were significantly higher in iBAT compared with visceral and subcutaneous WAT (PRDM16: $P < 0.05$; all others: $P < 0.005$; Fig. 4*B*). Serpin3ak (serine or cysteine peptidase inhibitor, clade A, member 3K) and Psat1 (phosphoserine aminotransferase) are preferentially expressed in WAT (58). As expected, their mRNA levels were significantly higher in visceral and subcutaneous WAT than in iBAT ($P < 0.005$) (Fig. 4*C*). Cold exposure induced a steady decrease in Serpin3ak expression in iBAT and a transient increase in visceral and subcutaneous WAT. Surprisingly, Psat1 expression was induced in all three depots on *day 1* and *day 3* but then reverted to its basal level on *day 6*.

C/EBPα is an antimitotic protein and an important regulator of terminal adipocyte differentiation (60, 62). C/EBPα mRNA expression was rapidly induced by cold acclimatization in the two WAT depots, but not in iBAT (Fig. 4*C*).

β_3-Adrenoceptor plays a central role in the cold-induced emergence of brown adipocytes in "white" fat depots. The number of brown adipocytes occurring in WAT after cold acclimatization was blunted in the subcutaneous and the visceral WAT depots of cold-acclimatized β_3-KO mice ($P < 0.01$ in subcutaneous and visceral WAT depots; Fig. 1), suggesting that β_3-adrenoceptor plays a central role in the appearance of brown adipocytes in the WAT of 129Sv mice. We obtained similar results using WT and β_3-KO mice on a pure B6 genetic background (Supplemental Fig. S1).

Cold acclimatization induced β_3-adrenoceptor-mediated morphological changes in UL white adipocytes, as shown by a size reduction only in WT mice (Fig. 5*A*). Furthermore, EM disclosed cold-induced modifications in mitochondrial morphology in these cells (Fig. 6*A*), as demonstrated by the fact that their mitochondria were similar to those found in PL brown adipocytes, as described above.

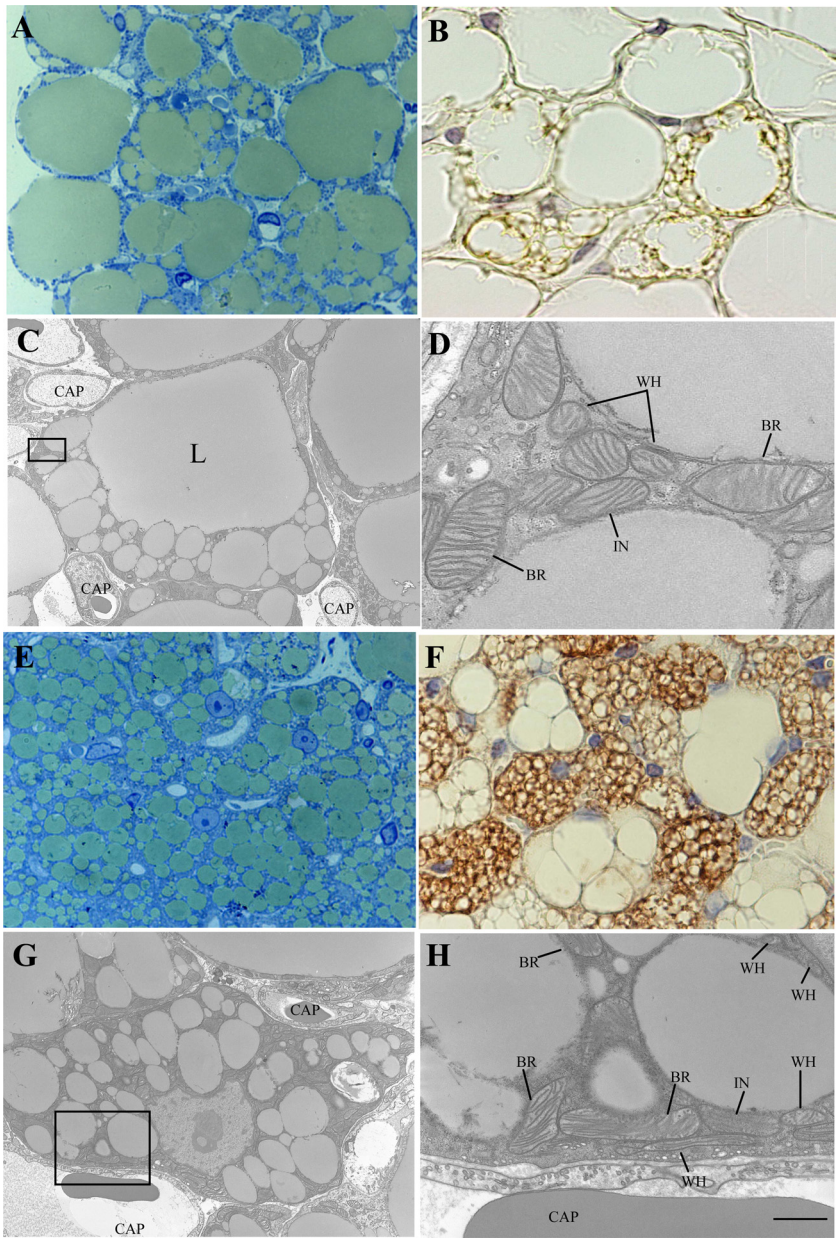

Fig. 2. Morphology, UCP1 immunoreactivity, and electron microscopy of classic and PL brown adipocytes. Representative light micrographs of subcutaneous WAT showing the morphology (*A*) and UCP1 immunoreactivity (*B*) of PL brown adipocytes and the morphology (*E*) and UCP1 immunoreactivity (*F*) of classic brown adipocytes. *C*: representative electron micrographs of PL brown adipocytes. Large lipid droplet (L) surrounded by several small lipid droplets in the cytoplasm of the same cell. *D*: enlargement of the area framed in *C*. Mitochondria with the typical features of classic mitochondria of brown adipocytes (BR) are found together with smaller "white"-like mitochondria (WH) and intermediate forms (IN). *G*: representative electron micrographs of classic ML brown adipocytes containing numerous small uniform lipid droplets. *H*: enlargement of the area framed in *G* showing BR, WH, and IN. CAP, capillaries. *A* and *E*: resin embedding, toluidine blue staining. *B* and *F*: paraffin embedding, UCP1 immunostaining with avidin-biotin-peroxidase complex method (brown). All are from subcutaneous fat of 129Sv mice. Scale bar: *A*, *B*, *E*, and *F* = 16 μm; *C* = 2.9 μm; *D* = 0.55 μm; *G* = 6 μm; *H* = 0.55 μm.

Gene expression studies demonstrated, as reported previously (40), that UCP1 mRNA expression in iBAT was induced by cold exposure in both WT and β_3-KO mice but that it was severely impaired in the visceral and subcutaneous WAT of β_3-KO mice. The expression of PGC-1α, C/EBPβ, PRDM16, Cidea (cell death-inducing DNA fragmentation factor, α-subunit-like effector A), and COX8b mRNA displayed a similar pattern (Fig. 4, *D* and *E*).

Serpin3ak and Psat1 expression was similar in WT and β_3-KO mice (Fig. 4*F*). C/EBPα expression was unaffected in all three depots in cold-exposed β_3-KO mice. Cyclin A1 (Ccna1), which is associated intimately with cell cycling and proliferation (49), was dramatically upregulated in iBAT in both WT and β_3-KO mice (Fig. 4*F*) but was not affected in visceral or subcutaneous depots. We conclude that induction of PGC-1α and UCP1 in visceral and subcutaneous adipose tissue requires functional β_3-adrenoceptors but not a significant induction of cell proliferation.

Cold acclimatization induces an increase in the density of preadipocytes in the white fat pads of β_3-KO but not of WT mice. In theory, the newly formed brown adipocytes appearing in WAT after cold exposure could derive from the development of preexisting preadipocytes. Therefore, we calculated the preadipocyte density in WT and β_3-KO mice either maintained at room temperature or acclimatized to the cold. We used EM to detect preadipocytes due to their well-described morphology (5, 19, 21, 50, 59) (see MATERIALS AND METHODS). Since we were interested in the phenomenon of cold-induced emergence of brown adipocyte in WAT, we focused on the fat pad exhibiting the largest number of newly formed brown adipocytes, i.e., the subcutaneous adipose tissue.

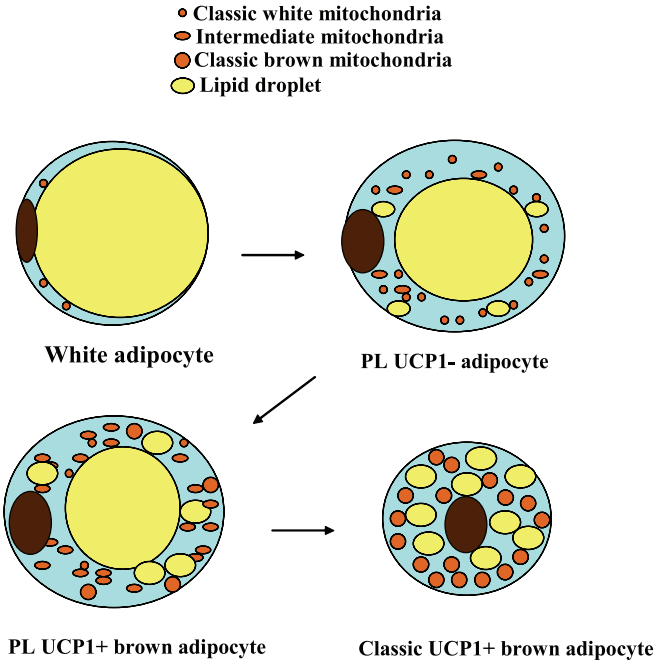

Fig. 3. Stages of white to brown transdifferentiation. Schematic showing of the transitional steps of transdifferentiation from white into brown adipocytes. Adipocytes were identified as unilocular (UL; containing a single large vacuole), PL (exhibiting a large vacuole surrounded by at least 5 small lipid droplets), or multilocular (ML; containing more than 5 small homogenous lipid droplets).

In control WT mice, preadipocytes appeared as poorly differentiated cells surrounded by a distinct external lamina (or basal membrane) and always located close to the extraluminal side of a capillary wall, as described previously (15, 19, 25, 45, 50, 59). In cold-acclimatized mice, preadipocytes in WAT had slightly more pronounced brown characteristics (5, 21, 45, 70), often with large mitochondria reminiscent of those found in early brown adipocyte differentiation (Fig. 6*B*), for instance, in the pericapillary areas of cold-exposed iBAT (Fig. 6*C*).

Mean preadipocyte density was found to be similar in cold-acclimatized and control WT mice, whereas it increased significantly (by ~17 times; $P < 0.001$) in cold-exposed β_3-KO mice (Fig. 5*B*). This increase, although detectable on quantitative EM, appears to be too small to be detected in gene expression studies (Fig. 4). Overall, these data suggest that cold acclimatization favors mainly the direct transformation of white into brown adipocytes (transdifferentiation) in WT animals, whereas in β_3-KO mice it promotes mainly the development of new brown adipocytes through the recruitment of preadipocytes.

β_1-Adrenoceptor controls brown preadipocyte density, and β_3-adrenoceptor controls white to brown adipocyte transdifferentiation. Cell culture studies have demonstrated that β_1-adrenoceptor is expressed in brown preadipocytes in the early stages of differentiation, whereas β_3-adrenoceptor appears at later stages (6–8). These data suggest that β_1-adrenoceptor could be responsible for the increase in the number of preadipocytes induced by the adrenergic stimulus, whereas β_3-adrenoceptor could be responsible for the functional thermogenic activity of differentiated brown adipocytes. Our findings support this notion and further suggest that the adrenergic stimulus (i.e., cold exposure) could act predominantly via β_1-adrenoceptor in β_3-KO mice, increasing the density of brown preadipocytes, and mainly via β_3-adrenoceptor in WT mice, inducing white to brown adipocyte transdifferentiation. To test this hypothesis, six groups of 129Sv mice were treated with saline or with β_1- or β_3-adrenoceptor agonists.

Since morphometry studies demonstrated that the number of brown adipocytes in the subcutaneous WAT of 129Sv mice peaked after 3–5 days of cold exposure (not shown), the β-adrenoceptor agonists were administered for 3 and 5 days. A significant brown adipocyte increase was seen only in the two groups receiving the β_3-adrenoceptor agonist (Fig. 5*C*). Interestingly, the proportion of PL brown adipocytes was significantly higher only in these mice (Fig. 5*D*).

As predicted, quantitative EM disclosed an increased density of preadipocytes only in mice treated with the β_1-adrenoceptor agonist ($P < 0.01$; Fig. 5*E*).

DISCUSSION

Cold acclimatization induces brown adipocytes in WAT (2, 16, 25, 35). The newly formed brown adipocytes are likely to be thermogenically active because they express the thermogenic protein UCP1 and have a phenotype similar to that of the brown adipocytes found in iBAT (10, 51). The observation that neither adipocyte number nor DNA content increases in white fat depots (24, 30, 46, 48) has suggested that cell proliferation mechanisms are not involved in this process.

The present finding that cold acclimatization did not increase WAT preadipocyte density lends support to this notion. We also observed an increased expression of the antimitotic gene C/EBPα (60, 62) and no change in Ccna1 (which is related to cell cycling and proliferation) (49). Consistent with these data, cold exposure, which is known to stimulate the proliferation of brown adipocytes in iBAT (9), did not affect the expression of C/EBPα and increased Ccna1 levels in this depot. Interesting genetic data by Coulter et al. (23) and Xue et al. (69) suggest that brown adipocyte formation is under the control of different mechanisms in WAT and iBAT, in line with the view of the separate developmental origin of these two cell types. Our data agree with this notion and argue for a direct transformation of white into brown adipocytes in cold-exposed mouse WAT. In fact, the significant increase of brown adipocytes after cold acclimatization occurred in the absence of any sign of apoptosis and of a significant increase in preadipocyte density. A novel EM finding of this work is the recognition of an intermediate form between white and brown adipocytes (PL adipocytes). These cells are UCP1 immunoreactive and show an intermediate form of lipid accumulation and a distinctive mitochondrioma (mitochondrial number and morphology, i.e., mitochondrial phenotype). They contain mitochondria subpopulations with morphologies ranging from classic brown (i.e., brown adipocytes) to classic white (i.e., white adipocytes). The possibility that these cells derive from a direct transformation of white adipocytes is underscored by the fact that several white UL adipocytes with a similar mitochondrioma were also detected in WAT after cold acclimatization. These cells may represent early stages of white to brown adipocyte transformation (see Fig. 3).

Of note, the mitochondrioma of mature adipocytes developing from the stroma vascular fraction of murine and human iBAT in primary cultures is comparable with the one described here; i.e., it consists of two mitochondrial subpopulations,

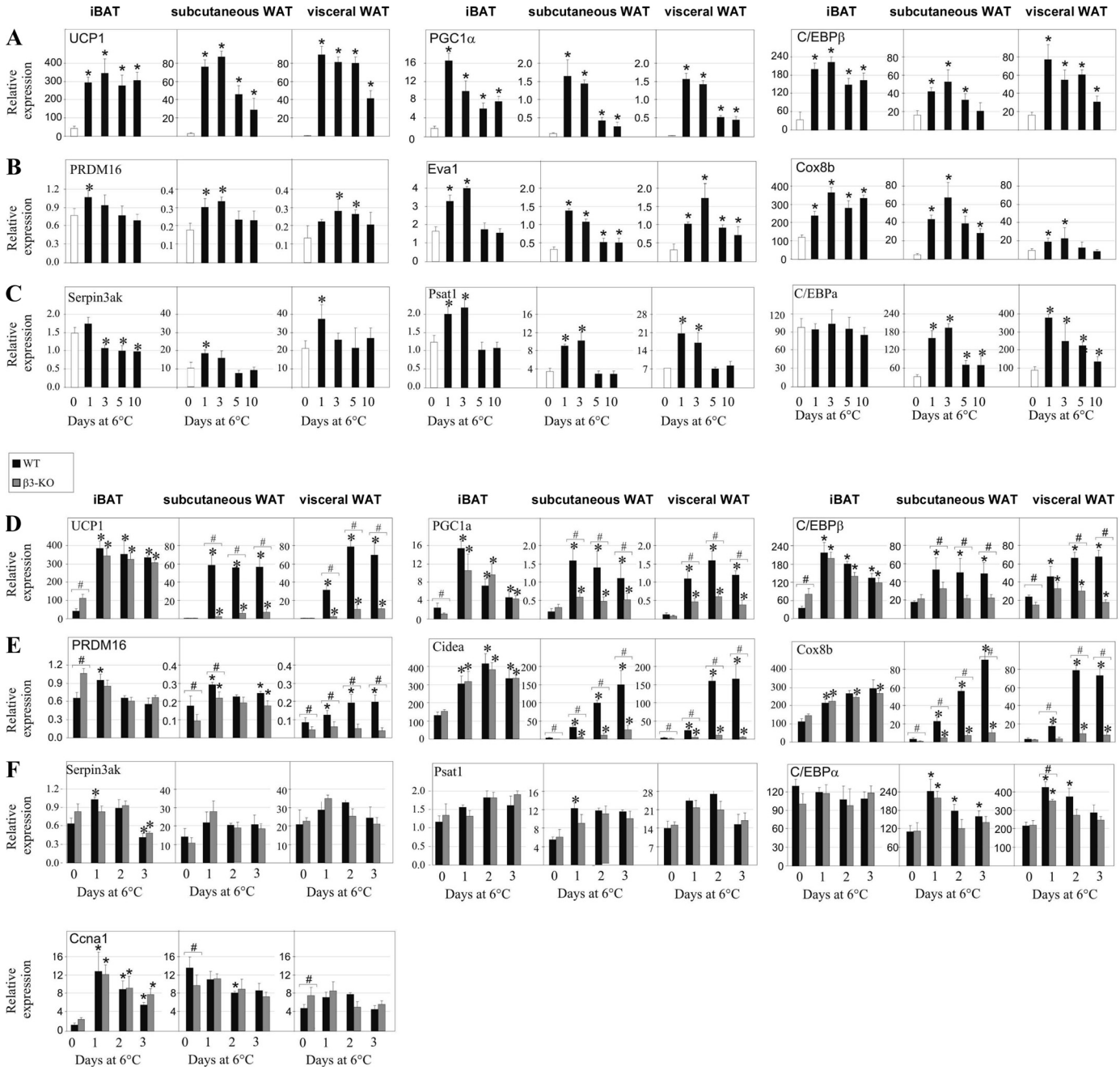

Fig. 4. Gene expression in fat depots. *A*, *B*, and *C*: female 129Sv mice were kept above room temperature (24 ± 1°C; controls) or at 6°C for 1, 3, 5, or 10 days. Interscapular brown adipose tissue (iBAT), subcutaneous WAT, and visceral WAT were dissected out and frozen in liquid nitrogen. RNA was extracted and cDNA synthesized from each mouse (4/group). Expression of UCP1, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), CCAAT/enhancer binding protein-β (C/EBPβ), PR domain containing 16 (PRDM16), cytochrome *c* oxidase, subunit VIIIb (Cox8b), epithelial V-like antigen 1 (Eva1), serine or cysteine peptidase inhibitor, clade A, member 3K (Serpina3ak), phosphoserine aminotransferase (Psat1), and C/EBPα was measured by RT-qPCR in duplicate and normalized to TATA box-binding protein. *D*, *E*, and *F*: female 129Sv WT and β_3-KO mice before and after 1, 2, or 3 days at 6°C. iBAT, subcutaneous WAT, and visceral WAT were dissected out and frozen in liquid nitrogen. RNA was extracted and cDNA synthesized from each mouse (4/group). Expression of UCP1, PGC-1α, C/EBPβ, PRDM16, Cox8b, cell death-inducing DNA fragmentation factor, α-subunit-like effector A (Cidea), Serpina3ak, Psat1, C/EBPα, and cyclin A1 (Ccna1) was measured by RT-qPCR in duplicate and normalized to TBP. Bars represent means ± SD. *Significant difference ($P < 0.05$) vs. mice kept at 24°C; #significant difference ($P < 0.05$) between β_3-KO and WT mice kept at the same temperature.

"brown" and "white" (11, 12). Addition of noradrenalin to these cells in culture induced the transformation of mitochondria into a more classic "brown" morphology (20).

Concomitant with the expected upregulation of the thermogenic genes, an unexpected transient increase in the levels of the genes selectively expressed in WAT was observed in subcutaneous and visceral depots. This finding may be explained by the massive presence of white/brown transition cells. The molecular mechanism controlling transdifferentiation seems to depend on a working β_3-adrenoceptor mechanism. This surmise is supported by the observation that both β_3-KO strains with different genetic backgrounds (B6 and

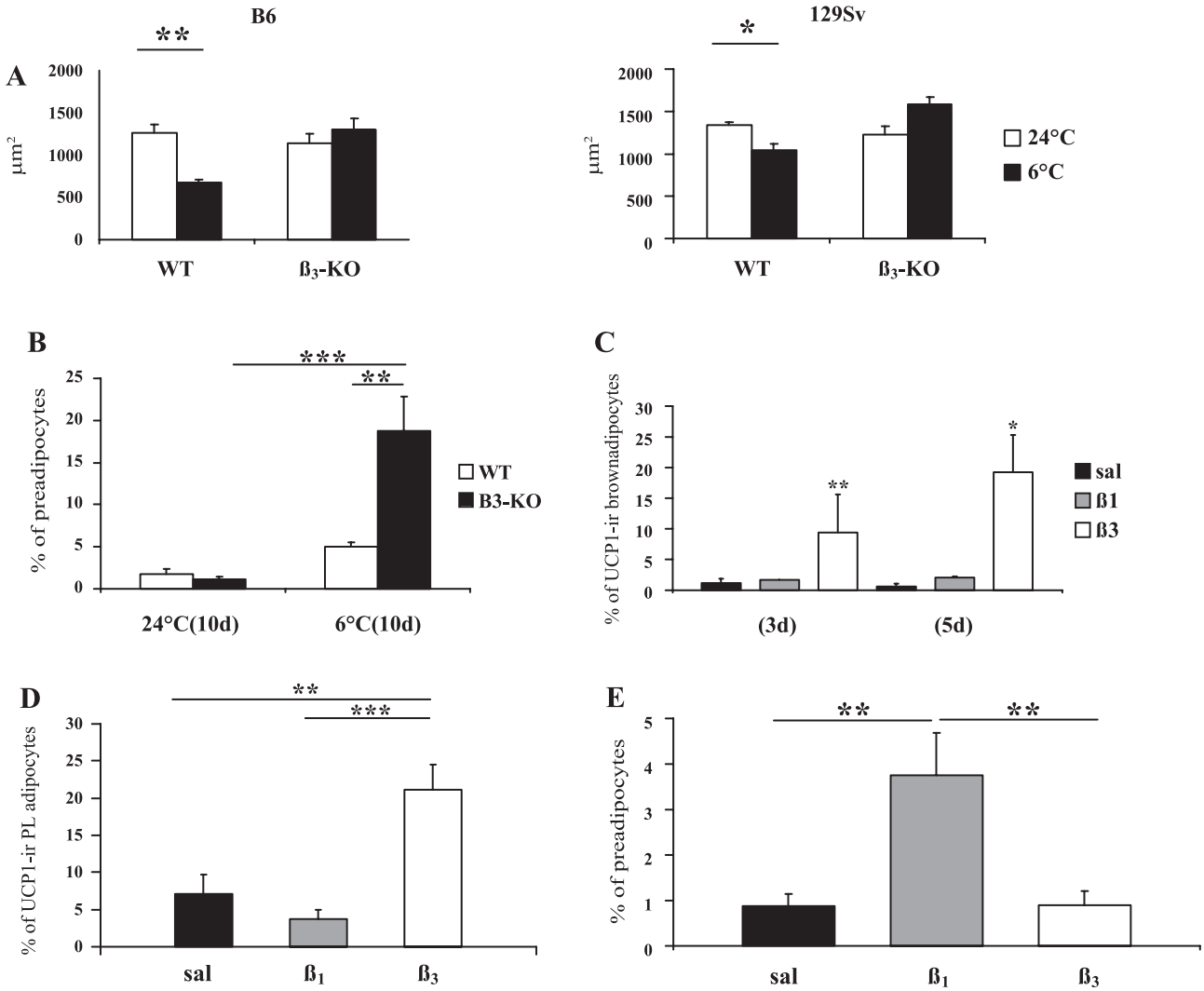

Fig. 5. Effect of cold exposure and of administration of selective β_1- and β_3-agonists on subcutaneous WAT. *A*: size of white adipocytes after cold acclimatization for 10 days; mean adipocyte area is reduced only in the 2 WT strains (B6 and 129Sv). Absence of β_3-adrenoceptor prevents reduction in adipocyte size. *B*: %preadipocytes (no./100 adipocytes). On quantitative electron microscopy, cold-induced preadipocyte density increased significantly only in β_3-KO mice. *C*: quantification of brown adipocytes (classic and PL) after administration of selective β_1- and β_3-agonists. Only administration of the β_3-agonist resulted in a significant brown adipocyte increase. *D*: quantification of PL brown adipocytes after administration of saline (sal) and selective β_1- and β_3-agonists. Only administration of the β_3-agonist resulted in a significant increase in PL brown adipocytes. *E*: proportion of preadipocytes after administration of sal and selective β_1- and β_3-agonists. Preadipocyte density increased only after β_1-agonist administration. Means ± SE. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

129Sv) used in this work showed an impaired reactivity of visceral and subcutaneous fat depots to cold exposure. This confirms previous data from our laboratories (40) and extends our findings to the subcutaneous depot and to different murine strains with pure backgrounds. The critical role of a functional β_3-adrenoceptor in this process is emphasized by our experiments with the β_3-adrenoceptor agonist CL316,24, whose administration resulted in the emergence of brown adipocytes whose morphology closely resembled that induced by cold acclimatization, including the absence of an increase in the density of brown preadipocytes. Significantly, we and other groups have demonstrated that CL316,243 induces the appearance of brown adipocytes in WAT, the vast majority of which (80–95%) were shown by bromodeoxyuridine labeling to arise in a proliferation-independent manner (34, 37).

Further supporting the hypothesis that transdifferentiation requires a functional β_3-adrenoceptor is the fact that cold acclimatization induced a significant increase in preadipocyte density in β_3-KO mice that was not seen in WT mice. These data and our in vivo experiments, documenting that administration of the β_1-adrenoceptor agonist xamoterol (31) induced an increase in preadipocyte density, agree with the hypothesis suggested by the in vitro studies that β_1-adrenoceptor is responsible for preadipocyte proliferation (7). The absence of β_3-adrenoceptor affected neither the functional expression of the thermogenic genes nor that of iBAT genes in iBAT, whereas it dramatically blunted the effect of cold acclimatization on their expression in WAT depots. Such dissociated control of the genes selectively expressed in brown vs. white adipose tissues by β_3-adrenoceptor agrees with a different origin of brown adipocytes in the two types of depot. Atit et al. (3) demonstrated that the central dermomyotome gives rise to both muscle and iBAT, but not WAT. In line with these results, recent work showed that iBAT brown preadipocytes express a

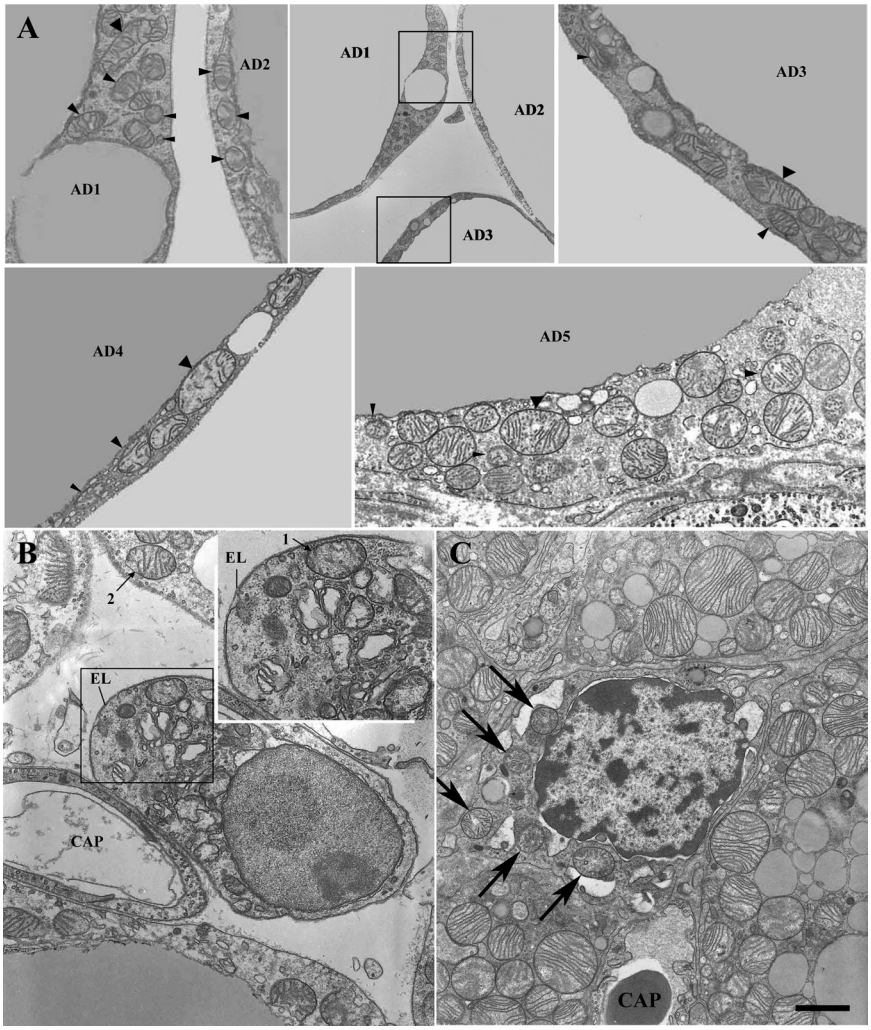

Fig. 6. Electron microscopic study of cold-acclimatized tissue. *A*: early steps of white to brown transdifferentiation. UL white adipocytes from subcutaneous WAT of 129Sv mice after cold acclimatization showing mitochondria with a variable size and morphology: small with "white" features (small arrowheads), intermediate (medium-sized arrowheads), and large with "brown" features (large arrowheads). Five different adipocytes (AD1–AD5) are shown. Framed areas are enlarged on either side. *B*: representative electron micrograph of preadipocytes in the pericapillary area of 129Sv mouse subcutaneous WAT kept at 6°C for 10 days. Preadipocytes are the only pericapillary cells with a high nuclear/cytoplasmic ratio and a distinct external lamina (EL). *B*, *inset*: enlargement of framed area. Note that mitochondrial morphology (*arrow 1*) is similar to that of surrounding ML brown adipocytes (*arrow 2*). *C*: a preadipocyte in iBAT after cold acclimatization. Representative electron micrograph of a preadipocyte in the pericapillary area. Note the high nuclear/cytoplasmic ratio, the EL, and the abundance of large mitochondria (arrows) in the early stage of differentiation reminiscent of the classic mitochondrial morphology seen in the mature brown adipocytes surrounding the preadipocyte. Scale bar: $A = 1.9$ μm; $B = 1.07$ μm; $C = 1.8$ μm.

wide range of muscle-related genes (63). In vivo mapping data, showing that the iBAT brown adipocytes but not the brown fat cells that emerged in the WAT of mice treated with the β_3-adrenoceptor agonist derive from Myf5-expressing cells, support the notion of different pathways of brown adipogenesis in WAT and iBAT (57). These data are in total agreement with those in the present study showing that, after cold exposure or β_3-adrenoceptor agonist administration, the newly formed brown adipocytes in iBAT conceivably derive from brown preadipocytes, whereas the new brown adipocytes appearing in WAT, in the absence of morphological and molecular signs of apoptosis, have a different origin, i.e., derived from the transdifferentiation of white into brown adipocytes. A remodeling of the adipose tissues by a transdifferentiation process resembling the one described in the present work could also take place in vivo following targeted deletion of the gene encoding RIIb, which induces a compensatory high expression of RIa and activation of cAMP-dependent PKA (26). Suppression of 4E binding protein-1, which inhibits PGC-1α translation, also induces adipose tissue remodeling (66). Interestingly, RIIb-KO and 4E binding protein-1-KO mice are lean and obesity resistant.

PPARγ (peroxisome proliferator-activated receptor-γ) agonists are used as insulin sensitizers, and their administration to rats and dogs induces a remodeling of the adipose tissues (65). In vitro experiments with human white adipocytes suggest that forced expression of PGC-1α or administration of PPARγ agonists can induce UCP1 expression (i.e., white to brown transdifferentiation) in these cells (64).

Transdifferentiation is a process whereby a mature differentiated cell transforms into a new phenotype with a different morphology and physiology without going through dedifferentiation (29). Adipocytes seem to possess this physiological property. We have previously found evidence for physiological, reversible, adipoepithelial transdifferentiation in the mouse mammary gland (28, 47). The above considerations, together with recent data disclosing that several discrete depots of metabolically active iBAT are found in adult humans (27, 50a, 67, 68, 70), suggest that white to brown adipocyte transdifferentiation could be of great interest for the treatment of diabetes and obesity, especially in light of the considerable body of data documenting an antidiabetic and antiobesity effect of brown adipocytes (1, 4, 36, 43).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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