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A Reservoir of Brown Adipocyte Progenitors in Human Skeletal Muscle

MIHAELA CRISAN,[a,b] LOUIS CASTEILLA,[a,c] LORENZ LEHR,[d] MAMEN CARMONA,[c] ARIANE PAOLONI-GIACOBINO,[e] SOLOMON YAP,[a] BIN SUN,[a] BERTRAND LÉGER,[f] ALISON LOGAR,[a] LUC PÉNICAUD,[c] PATRICK SCHRAUWEN,[g] DAVID CAMERON-SMITH,[h] AARON PAUL RUSSELL,[h] BRUNO PÉAULT,[a,b] JEAN-PAUL GIACOBINO[a]

[a]Stem Cell Research Center, Children's Hospital, [b]McGowan Institute for Regenerative Medicine, and [e]Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; [c]UMR 5241 CNRS-UPS, IFR 31, Institut Louis Bugnard, Toulouse, France; [d]Department of Cell Physiology and Metabolism, Centre Médical Universitaire, University of Geneva, Geneva, Switzerland; [f]Clinique Romande de Réadaptation, SUVA Care, Sion, Switzerland; [g]Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands; [h]School of Exercise and Nutrition Sciences, Deakin University, Burwood, Australia

Key Words. Brown adipocytes • Human muscle

ABSTRACT

Brown adipose tissue uncoupling protein-1 (UCP1) plays a major role in the control of energy balance in rodents. It has long been thought, however, that there is no physiologically relevant UCP1 expression in adult humans. In this study we show, using an original approach consisting of sorting cells from various tissues and differentiating them in an adipogenic medium, that a stationary population of skeletal muscle cells expressing the CD34 surface protein can differentiate in vitro into genuine brown adipocytes with a high level of UCP1 expression and uncoupled respiration. These cells can be expanded in culture, and their UCP1 mRNA expression is strongly increased by cell-permeating cAMP derivatives and a peroxisome-proliferator-activated receptor-γ (PPARγ) agonist. Furthermore, UCP1 mRNA was detected in the skeletal muscle of adult humans, and its expression was increased in vivo by PPARγ agonist treatment. All the studies concerning UCP1 expression in adult humans have until now been focused on the white adipose tissue. Here we show for the first time the existence in human skeletal muscle and the prospective isolation of progenitor cells with a high potential for UCP1 expression. The discovery of this reservoir generates a new hope of treating obesity by acting on energy dissipation. STEM CELLS *2008;26:2425–2433*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Uncoupling protein-1 (UCP1) is the main effector of adaptative thermogenesis in rodents. Specifically expressed in brown adipose tissue (BAT) mitochondria, UCP1 acts as an uncoupler of oxidative phosphorylation and dissipates energy as heat. BAT thermogenesis in rodents is increased upon exposure to low temperature or as a result of overeating. It is controlled by the sympathetic nervous system that stimulates mitochondriogenesis and UCP1 expression and activity [1]. BAT therefore plays an important role in the maintenance of body temperature and energy balance [2, 3]. In rodents, ectopic brown adipocytes can also be found, outside the BAT, in white adipose tissue (WAT) depots [4]. Their emergence is induced by cold acclimation [5–7] and β_3-adrenoceptor agonist administration [7, 8]. Ectopic WAT brown adipocytes might act in synergy with the BAT to prevent obesity [7, 8].

In humans, typical BAT expressing UCP1 is present in neonates but was considered until recently to disappear early in life [9, 10]. In adult humans few brown adipocyte progenitors still exist in the WAT, which can be induced to differentiate into UCP1-expressing cells in vitro by β-adrenoceptor agonists [11] and thiazolidinediones [12] or in vivo in the vicinity of catecholamine-secreting pheochromocytomas [13]. Because of its scarcity, however, this candidate dormant population cannot be considered a reliable source for brown adipocyte reappearance in humans.

Recently, however, there has been a resurgence of interest in the hypothesis that BAT might play a role in adult humans.

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Correspondence: Jean-Paul Giacobino, M.D., 16 rue Jean-Sénebier, 1205 Geneva, Switzerland. Telephone: 4122-310-62-84; Fax: 4122-379-52-28; e-mail: jean-paul.giacobino@medecine.unige.ch Received April 3, 2008; accepted for publication June 25, 2008; first published online in STEM CELLS *EXPRESS* July 10, 2008. ©AlphaMed Press 1066-5099/2008/$30.00/0 doi: 10.1634/stemcells.2008-0325

Indeed, fluorodeoxyglucose positron emission tomography studies allowed visualizing in humans highly dynamic adipose tissue depots in the upper part of the body. Their metabolism was stimulated by cold exposure and inhibited by β-blockers. These depots were proposed to represent BAT that had been undetected until now [14]. Many more studies are needed to define ex vivo the metabolic profile and in vivo the possible physiological role of these BAT depots in adult humans.

Furthermore, a study performed in mice showed, recently, the presence of ectopic brown adipocytes expressing UCP1 in the skeletal muscle. This study also showed that the number of UCP1-positive cells and the level of UCP1 mRNA in the muscle were higher in obesity-resistant 129S6 mice than in obesity-prone C57BL6 mice and suggested that the muscle ectopic BAT deposits reflect a genetic mechanism of protection against weight gain [15]. However, the observation that neither 129S6 nor C57BL6 mice respond to high-fat feeding by an upregulation of UCP1 expression in their muscle does not support this hypothesis [16]. In a different field, liver X receptor-null mice have been reported to be obesity-resistant. Intriguingly, an upregulation of UCP1 expression and an uncoupled oxidative phosphorylation were observed in their skeletal muscle [17].

The novel uncoupling proteins UCP2 and UCP3, abundantly expressed in human tissues, were first considered as thermogenic proteins [18, 19]. It is, however, now generally admitted that they are not involved in adaptative thermogenesis [20]. The gold-standard thermogenic uncoupling protein, therefore, remains UCP1.

Before the review of Nedergaard et al. [14] reporting the existence of BAT-like depots in adult humans and the study of Almind et al. [15] showing the occurrence of brown adipocyte ectopic depots outside the WAT, we had started an independent study with the aim of identifying in human tissues possible progenitors of brown adipocytes that might differentiate in culture into mature cells expressing UCP1.

It has been shown that subsets of vascular cells (i.e., endothelial cells and pericytes) are a source of multilineage progenitors in human tissues [21–23]. The surface antigens CD34, which is expressed by both hematopoietic progenitors and vascular endothelial cells [24], and CD146, a marker of the pericytes that surround the endothelial layer in capillaries and microvessels [25], were chosen to sort progenitors and endothelial cells on one hand and pericytes on the other. The potential of each of these cell populations, sorted from various human fetal and adult tissues and grown in an adipogenic medium, to differentiate in culture into brown adipocytes was tested. It was found that CD34+ cells from skeletal muscle but not from WAT display the unique capacity to differentiate in vitro into genuine brown adipocytes with a high level of UCP1 expression. The dormant muscle CD34+ cell population might prove a reliable target for brown adipocyte resurgence in humans.

MATERIALS AND METHODS

Materials

All organic and inorganic chemicals of analytical or molecular biology grade were purchased from Sigma-Aldrich (St. Louis, http://www.sigmaaldrich.com) and Gibco-BRL (New York, http://www.gibcobrl.com).

Human Tissues

Human fetal tissues were obtained anonymously, following spontaneous, voluntary, or therapeutic terminations of pregnancy, from Magee-Womens Hospital, University of Pittsburgh, in compliance with the institutional review board protocol. Developmental age (16–24 weeks of gestation) was estimated by measuring foot length. Informed consent to the use of fetal tissues was obtained from the patients in all instances. Adult human discarded abdominal subcutaneous WAT, originating from 45–55-year-old patients undergoing plastic surgery performed 1 year after gastric bypass, was kindly provided by Dr. Peter Rubin (Division of Plastic Surgery, University of Pittsburgh). The adult skeletal muscle used for cell sorting was obtained post mortem from 50–78-year-old donors. The adult skeletal muscle used for the first group of reverse transcription (RT)-polymerase chain reaction (PCR) studies was obtained from the rectus abdominus during surgery for either lap banding, inguinal hernia, or hysterectomy of 10 lean male and female subjects. All subjects agreed to donate muscle samples during their operations, and the protocol was approved by the Medical Ethical Review Committee of Deakin University. The average age was 45 ± 3 years, and the average body mass index was 22.2 ± 0.8. The adult skeletal muscle used for the second group of RT-PCR studies was obtained from the vastus lateralis of seven obese type 2 diabetic male and female subjects before and after 8 weeks of treatment with rosiglitazone (two doses of 4 mg each per day). The average age was 63 ± 4 years, and the average body mass index was 29.9 ± 3.8. The complete clinical profile of the patients has been described in a previous publication [26]. All subjects agreed to donate muscle samples, and the protocol was approved by the Medical Ethical Review Committee of Maastricht University.

Mice

Animals were treated in accordance with the Centre Médical Universitaire (Geneva, Switzerland) institutional guidelines. They were housed individually and kept on a 12-hour light-dark cycle in a temperature-controlled room at 24°C. They were allowed ad libitum access to water and a standard laboratory chow. The interscapular BAT of 4–6-week-old male 129 Sv/ev mice was excised, and their precursor cells were isolated and cultured as previously described [27].

Immunohistochemistry

Fresh fetal and adult tissues were gradually frozen by immersion in isopentane cooled in liquid nitrogen. Five- to 7-μm sections were cut on a cryostat (Microm HM 505 E [Mikron Instrument Company, Inc., Oakland, NJ, http://www.mikron.com]), fixed with 50% acetone and 50% methanol, dried for 5 minutes at room temperature, and then washed three times for 5 minutes in phosphate-buffered saline. Nonspecific binding sites were blocked with 5% goat serum for 1 hour at room temperature. Sections were incubated overnight at 4°C with a CD34 mouse anti-human antibody (1:50; AbD Serotec, Raleigh, NC, http://www.ab-direct.com) and then, after rinsing, for 1 hour at room temperature with a secondary goat anti-mouse biotinylated antibody (1:1,000; Dako, Glostrup, Denmark, http://www.dako.com) and for 30 minutes at room temperature with streptavidin-Cy3 (1:1,000; Sigma-Aldrich) or for 2 hours at room temperature with a conjugated CD146-Alexa 488 mouse anti-human antibody (1:200; Chemicon, Temecula, CA, http://www.chemicon.com). Nuclei were stained with 4′, 6-diamino-2-phenylindole dihydrochloride (1:2,000; Molecular Probes, Eugene, OR, http://probes.invitrogen.com) for 5 minutes at room temperature. An isotype-matched negative control was performed with each immunostaining.

Flow Cytometry

The vascular cells of fetal skeletal muscle, pancreas, lung, and liver, as well as those of adult muscle and WAT, were analyzed by flow cytometry. Fresh fetal and adult muscle, as well as fetal pancreas, lung, and liver tissues, were cut into small pieces with a scalpel in Dulbecco's modified Eagle's medium (DMEM) high-glucose containing 20% fetal bovine serum (FBS), 1% penicillin-streptomycin (PS), and collagenases IA-S, II-S, and IV-S (1 mg/ml) and then incubated at 37°C for 75 minutes (fetal tissues) or 90 minutes (adult tissues) with constant stirring. Final cell dissociation was achieved between ground-glass slides. Cells were washed with phosphate-buffered saline and centrifuged for 5 minutes at 350*g*. They were resuspended in DMEM, 20% FBS; filtered at 100 μm; stained with trypan blue; and counted after dead cell exclusion. The WAT stroma vascular fraction was prepared by collagenase digestion according to Champigny et al. [28]. Cells (10^5 cells for analysis and approximately 30×10^6 cells for sorting) were incubated with one of the following directly coupled mouse anti-human antibodies: CD45-APC Cy7 (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), CD56-PE Cy7 (1:100; BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml), CD34-PE (1:100; Dako), and CD146-fluorescein isothiocyanate (FITC) (1:100; AbD Serotec) in 1 ml of DMEM, 20% FBS, 1% PS, at 4°C for 15 minutes. After washing and centrifugation, cells were incubated for 30 minutes with 7-aminoactinomycin D (1:100; BD Pharmingen) for dead cell exclusion, filtered at 70 μm, and run on a FACSAria flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). As negative controls, cell aliquots were incubated with isotype-matched mouse IgGs conjugated to APC Cy7 (1:100; BD Pharmingen), PE Cy7 (1:100; BD Pharmingen), PE (1:100; Chemicon), and FITC (1:100; United States Biological, Swampscott, Massachusetts, http://www.usbio.net) under the same conditions.

Cell Culture

Cells were seeded at 2×10^4 cells per cm^2 in 0.2% gelatin-coated plates and cultured until confluence (4–6 days) at 37°C in EGM2 medium (Cambrex, Walkersville, MD, http://www.cambrex.com) and until differentiation (8–12 more days) in a modification of the adipogenic medium described by Rodriguez et al. [29] consisting of DMEM-Ham's F-12 medium containing 0.86 μM insulin, 10 μg/ml transferrin, 0.2 nM triiodothyronine, 1 μM rosiglitazone (GlaxoSmithKline, Research Triangle Park, NC, http://www.gsk.com), 100 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethazone, and 1% PS. For cell expansion studies, confluent cells grown in EGM2 medium only were detached by treatment with trypsin-EDTA for 3–5 minutes at 37°C and then split 1:3 and cultured as described above. Human white adipocytes in primary culture used in the oxymetry studies were obtained as previously described [30].

RT-PCR

Total cell RNA was prepared using the NucleoSpin RNAII kit (Clontech, Palo Alto, CA, http://www.clontech.com) or Extract-all solution (Eurobio, Les Ulis, France, http://www.eurobio.fr) and quantified by biophotometry (BioPhotometer; Eppendorf AG, Hamburg, Germany, http://www.eppendorf.com). Oligo(dT)-primed first-strand cDNA was synthesized using the Superscript II RNase H Reverse Transcription kit (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and oligo(dT) primers or the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) and random primers. Quantitative real-time RT-PCR was performed using the ABI rapid thermal cycler system and a SYBR Green PCR master mix (Applied Biosystems). Cyclophilin A was used as a control to account for any variations due to the efficiency of the reverse transcription. The upstream and downstream oligonucleotide primers were chosen on both sides of an intron to prevent amplification of contaminating genomic DNA. The primers used for quantitative RT-PCR in human cells and in mouse brown adipocytes are described in supplemental online Table 1, those used for quantitative RT-PCR in human skeletal muscle are described in supplemental online Table 2, and those used for analytical RT-PCR are described in supplemental online Table 3.

Validation of the Human UCP1 Amplicon

The PCR-amplified fragment was cloned into the pCR2.1-TOPO vector through the TOPO-TA cloning system (Invitrogen), and purification of color-selected colonies was performed using the Qiaprep Spin Miniprep (Qiagen, Hilden, Germany, http://www1.qiagen.com). Sequences were determined with oligonucleotide M13 Reverse on the pCRII-TOPO vector using the Applied Biosystems Big Dye sequencing kit on an ABI 3700 automated sequencer (Applied Biosystems).

Western Blots

Cultured cells were collected with a rubber policeman in 200 μl of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1:200 protease inhibitor cocktail [Sigma-Aldrich], and 50 mM Tris-HCl, pH 8.0). Human BAT and skeletal muscle were homogenized in the above RIPA buffer. The protein content was determined according to the technique of Lowry et al. [31]. Western blots were performed as previously described [32]. The UCP1 protein was detected using a 1:500 diluted rabbit anti-mouse UCP1 polyclonal primary antibody generously provided by Dr. B. Cannon (Stockholm, Sweden). This antibody had been raised against the C-terminal decapeptide of mouse UCP1, which shares 80% identity with human UCP1 and 0% and 10% identities with human UCP2 and UCP3, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein was detected using a 1:5,000 diluted mouse anti-mouse GAPDH monoclonal primary antibody (Chemicon). Also used were 1:5,000 diluted goat anti-rabbit or anti-mouse peroxidase-labeled secondary antibodies (Sigma-Aldrich or Bio-Rad [Hercules, CA, http://www.bio-rad.com]). A SeeBlue Plus 2 Pre-stained Standard Ladder (Invitrogen) was used. Protein signals were detected by chemiluminescence using a standard ECL kit and developed on a Hyperfilm ECL film (GE Healthcare, Chicago, http://www.gehealthcare.com).

High-Resolution O_2 Consumption Measurement

Oxygen consumption was measured using a two-injection-chamber respirometer equipped with a Peltier thermostat, Clark-type electrodes, and integrated electromagnetic stirrers (Oroboros Oxygraph; Oroboros, Innsbruck, Austria, http://www.mitophysiology.org). Measurements were performed at 37°C with continuous stirring in 2 ml of DMEM-Ham's F-12 medium, 10% newborn calf serum. Under these conditions, the serum provided the fatty acids necessary to sustain UCP1 uncoupling activity [10]. Before each O_2 consumption measurement, the medium in the chambers was equilibrated with air for 30 minutes, and freshly trypsinized cells were transferred into the respirometer glass chambers. After steady-state respiratory flux was observed, ATP synthase was inhibited with oligomycin (0.25–0.5 mg/l), and cells were titrated with the uncoupler carbonyl cyanide 3-chlorophenylhydrazone up to optimum concentrations in the range of 1–2 μM. The respiratory chain was inhibited by antimycin A (1 μg/ml). Oxygen consumption was calculated using DataGraph software (Oroboros).

Microarray Analysis

The total RNA of fetal muscle CD34+ cells expanded in culture for up to three passages (4 weeks) and of human muscle biopsies were prepared as described above. The quality assurance measurements, the preparation of the cRNA targets, and the microarray analyses using the Illumina Human WG-6 BeadChip (Illumina Inc., San Diego, http://www.illumina.com) were performed by Expression Analysis (Durham, NC, http://www.expressionanalysis.com). BeadStudio nonparametric methods were used for the computation of detection *p* values.

Statistical Analysis

Data are expressed as means ± SEM. Significances were evaluated using the unpaired Student's *t* test. A paired Student's *t* test was used to determine the effects of rosiglitazone on human skeletal muscle UCP1 mRNA levels. Significances were set at $p < .05$.

RESULTS

Sorting of Muscle Vascular Cells

In fetal skeletal muscle, CD34 and CD146 were found, by immunohistochemistry, to be expressed at the surfaces of endothelial cells and pericytes, respectively, although CD34 was also expressed by cells scattered in the intermyofibrillar space (Fig. 1A). A similar distribution of CD34+ and CD146+ cells was observed in adult skeletal muscle (not shown). We next sorted

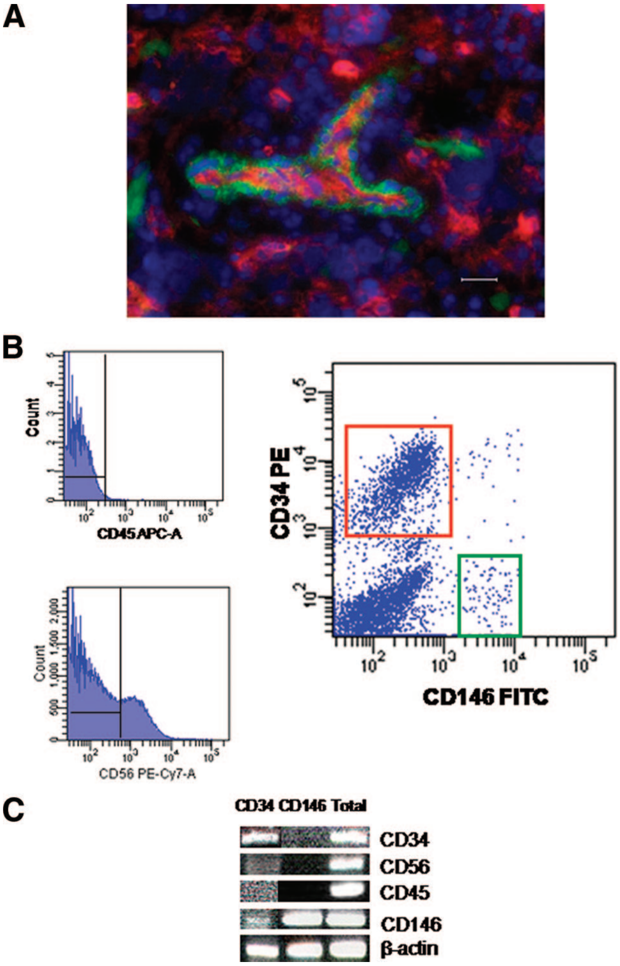

Figure 1. Immunohistochemical description and fluorescence-activated cell sorting analysis and sorting of vascular cells in human fetal muscle. **(A):** In a small vessel longitudinal section, CD146+ pericytes (green) surround CD34+ endothelial cells (red). Scale bar = 50 μM. **(B):** CD34+/CD146− and CD34−/CD146+ cell purification. Dissociated cells were stained with PE-anti-CD34, FITC-anti-CD146, PE-Cy7-anti-CD56, and APC-Cy7-anti-CD45 antibodies and run on a FACSAria cell sorter. Following exclusion of CD45+ and CD56+ cells (left panels), cells inside the CD34+ or CD146+ gates were sorted. **(C):** Reverse transcription-polymerase chain reaction analysis of CD34+/CD146−/CD45−/CD56− (CD34), CD34−/CD146+/CD45−/CD56− (CD146), and total nonsorted cells. β-actin mRNA was measured as a control. Abbreviation: FITC, fluorescein isothiocyanate.

vascular cells from seven independent fetal muscles (16–24 weeks of gestation) using multicolor fluorescence-activated cell sorting (FACS). Hematopoietic (CD45+) cells were first gated out, as were myogenic progenitors (CD56+). Then, endothelial cells (CD34+/CD146−) and pericytes (CD34−/146+) were sorted. The CD34+/CD146−/CD45−/CD56− were thereafter designated CD34+ cells, and the CD34−/CD146+/CD45−/CD56− were designated CD 146+ cells. The CD34+ cells amounted to 8% ± 1% of the starting fetal muscle cell population (Fig. 1B) and were shown by RT-PCR analysis not to be contaminated by detectable CD45+ hematopoietic or CD56+ myogenic cells (Fig. 1C).

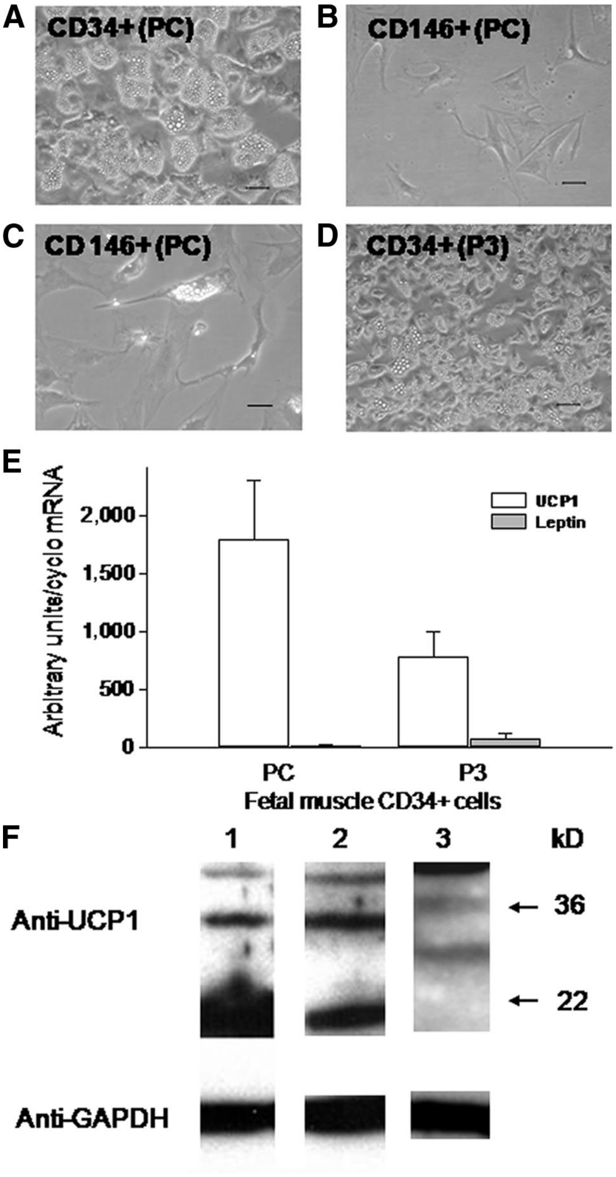

Figure 2. Culture under adipogenic conditions of cells sorted from human fetal muscle. CD34+ **(A)** or CD146+ **(B, C)** cells in PC and CD34+ **(D)** cells expanded in culture up to P3. All the cells were grown for 4–6 days in EGM2 medium and then placed for 8–12 days in the adipogenic medium described in Materials and Methods. Numerous adipocytes developed from CD34+ **(A, D)** but not from CD146+ cells **(B, C)**. Shown are phase-contrast images. Scale bar = 50 μm. **(E):** Quantitative reverse transcription polymerase chain reaction determination of UCP1 (open columns) and leptin (gray columns) mRNA expression in CD34+ cells in PC or expanded up to P3. The results are the mean ± SEM of arbitrary values normalized using the corresponding cyclophilin A values. n = 4–7. **(F):** Representative Western blot analysis of UCP1 and GAPDH proteins in tissue or whole cell extracts. Shown are interscapular brown adipose tissue of a 19-week fetus (lane 1) and CD34+ cells in PC (lane 2) or skeletal muscle of an adult human (lane 3). On each lane, 25 μg of proteins was loaded. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; kD, kilodaltons; P3, passage 3; PC, primary culture; UCP1, uncoupling protein-1.

The sorted cells were grown under conditions that sustained optimal white adipocyte differentiation in WAT primary cultures (i.e., 4–6 days in EGM2 medium and 8–12 days in the adipogenic medium, as described in Materials and Methods). Virtually all sorted fetal muscle CD34+ cells differentiated into adipocyte-like multilocular cells (Fig. 2A). It is noteworthy that in cell culture, the multilocular structure is shared by white and brown adipocytes. In contrast, fetal muscle CD146+ cells grew very slowly under the conditions described above. They did not reach cell confluence and displayed a pericyte-like appearance

Table 1. Levels of expressions of selected gene mRNAs

Selected mRNAs	CD34+ cells	Human muscle biopsies
UCP1	94	NS
mTFA	413	205
PPARγ	3,326	84
PGC-1α	137	619
COX IV	13,082	13,407
SDH	2,390	5,187
CPT1B	99	639
ACAD	1,032	141
ACADM	599	1,640
Myogenin	NS	267
Myf5	NS	21
MyoD	NS	12
Cidea	337	NS

The data are expressed as the average Illumina signal in arbitrary units. The detection p values were $<.01$.
Abbreviations: ACAD, acyl-coenzyme A dehydrogenase long chain; ACADM, C-4 to C-12 straight chain; COX IV, cytochrome oxidase IV; CPT1B, carnitine palmitoyltransferase 1B; mtTFA, mitochondrial transcription factor A; Myf5, myogenic factor 5; MyoD1, myogenic differentiation 1; NS, not significant; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PPARγ, peroxisome proliferator-activated receptor-γ; SDH, succinate dehydrogenase; UCP1, uncoupling protein-1.

characterized by a large size, spread-out shape, and irregular borders (Fig. 2B, 2C). Occasional multilocular cells could be detected (Fig. 2C) The morphology of CD34+ cells expanded in culture for up to three passages (4 weeks) under the conditions described above was similar to that observed in primary culture, although the size of mature adipocytes was smaller (Fig. 2C).

UCP1 Expression in Cultivated CD34+ Cells

The remarkable adipocyte-like differentiation of fetal muscle CD34+ cells was an incentive for further characterization. Strikingly, quantitative RT-PCR revealed a high level of UCP1 mRNA in these cells. The mean UCP1 mRNA level normalized with cyclophilin A was 1,797 ± 510 arbitrary units, corresponding to a Ct of 22 for 25 ng of cDNA in the assay (Fig. 2E). For comparison, the mean UCP1 mRNA level normalized with cyclophilin A in mouse brown adipocytes differentiated in culture was 7,715 ± 2,649 ($n = 10$) arbitrary units (not shown). Therefore, the level of UCP1 mRNA in human CD34+ cells amounted to almost one-fourth that in mouse brown adipocytes in culture. It should be noted that the human fetus BAT could not be used as a positive control for quantitative RT-PCR analysis since, because of the time elapsed after the termination of the pregnancy, the risk of RNA degradation was too high. The amplicon was cloned and sequenced and found to be 100% homologous to human *UCP1* gene. In fetal muscle CD34+ cells expanded up to passage 3, a high UCP1 mRNA expression, amounting to 43% of that detected in primary cultured cells, was still observed. UCP1 mRNA expression was not detected in nondifferentiated fetal muscle CD34+ cells or in CD146+ cells in primary culture. The level of leptin mRNA was 9.9 ± 5.5 and 71 ± 52 arbitrary units in primary cultured and expanded cells, respectively (Fig. 2E).

Further Phenotyping of the CD34+ Cells

To better characterize the gene expression pattern of the fetal muscle CD34+ cells expanded in culture, a microarray analysis was performed. The levels of expression of several representative gene mRNAs with significant detection p values ($p < .01$) are shown in Table 1 and compared with those in human muscle biopsies. The mRNAs of the genes coding for the following proteins were chosen: UCP1 as a reference; mitochondrial transcription factor A (mtTFA), peroxisome-proliferator-activated receptor-γ (PPARγ), and PPARγ coactivator-1α (PGC-1α), which are involved in the control of thermogenesis and mitochondriogenesis [33, 34]; enzymes of the mitochondrial respiratory chain cytochrome oxidase IV (COX IV) and succinate dehydrogenase (SDH); enzymes of the fatty acid degradation pathway carnitine palmitoyltransferase 1B (CPT1B), acyl-coenzyme A dehydrogenase long chain (ACAD), and C-4 to C-12 straight chain (ACADM); and the skeletal muscle markers myogenin, myogenic factor 5 (Myf5), and myogenic differentiation1 (MyoD1). Cidea, which is highly expressed in the BAT, where it acts as a suppressor of UCP1 activity [35], was chosen as a BAT marker. The accession numbers of the corresponding genes are shown in the supplemental online data.

UCP1 was significantly expressed in fetal muscle-expanded CD34+ cells but not in adult muscle biopsies. The levels of mRNA expressions of the selected genes in fetal muscle-expanded CD34+ cells are comparable with those of the adult muscle biopsies, with the exception of PGC-1α and CPT1B mRNAs, which are approximately 5-fold less expressed in the cells, and of the PPARγ and ACAD mRNAs, which are 40- and 7-fold less expressed, respectively, in the muscle. The muscle markers myogenin, Myf5, and MyoD1 mRNA were significantly expressed in the muscle but not in the cells, whereas the BAT marker Cidea mRNA was expressed in the cells but not in the muscle. No β_3-adrenoceptor mRNA could be detected in the microarray analysis. It is noteworthy, however, that β_3-adrenoceptor mRNA was detected by quantitative RT-PCR (arbitrary value, 0.084 ± 0.044 with cyclophilin A as a reference; $n = 4$) in fetal muscle CD34+ cells in primary culture. Measurements of mtTFA, PGC1-α, and COX IV mRNA content were also performed by quantitative RT-PCR to check the microarray data by another technique. The results were confirmatory, showing in fetal muscle CD34+ cells in primary culture, using cyclophilin A as a reference, high mtTFA, PGC1-α, and COX IV mRNA levels amounting to 306 ± 117, 385 ± 294, and 23,400 ± 10,300 arbitrary units ($n = 3$–4), respectively. The UCP1 protein, as assessed by Western blotting with an anti-mouse antibody cross-reacting with human UCP1 (80% identity), was as abundant in primary cultured fetal muscle CD34+ cells as in fetal BAT (Fig. 2F).

Uncoupling of Oxidative Phosphorylation

To get insight into the possible function of UCP1 in muscle-derived cells, mitochondrial respiration of isolated cultured human fetal muscle CD34+ cells and human adult white adipocytes was compared. Basal respiration was defined as the antimycin A-sensitive oxygen consumption. Uncoupled respiration was defined as the percentage of basal respiration insensitive to the ATP synthase blocker oligomycin. The ratios of uncoupled to total respiration were 47% ± 12% and 19% ± 2% in human fetal muscle CD34+ cells and adult white adipocytes, respectively (Fig. 3A).

Modulations of UCP1 Expression in Cultivated CD34+ Cells

UCP1 mRNA expression in fetal muscle CD34+ cells could be modulated by drug treatment. Cell-permeating cAMP derivatives strongly stimulated (7–8-fold) UCP1 mRNA expression in both primary cultured and expanded cells (Fig. 3B). PPARγ agonists increase UCP1 expression in rodent BAT [36]. Rosiglitazone, a PPARγ agonist, had no effect in primary culture cells but strongly stimulated (eightfold) UCP1 mRNA expression in expanded cells (Fig. 3C).

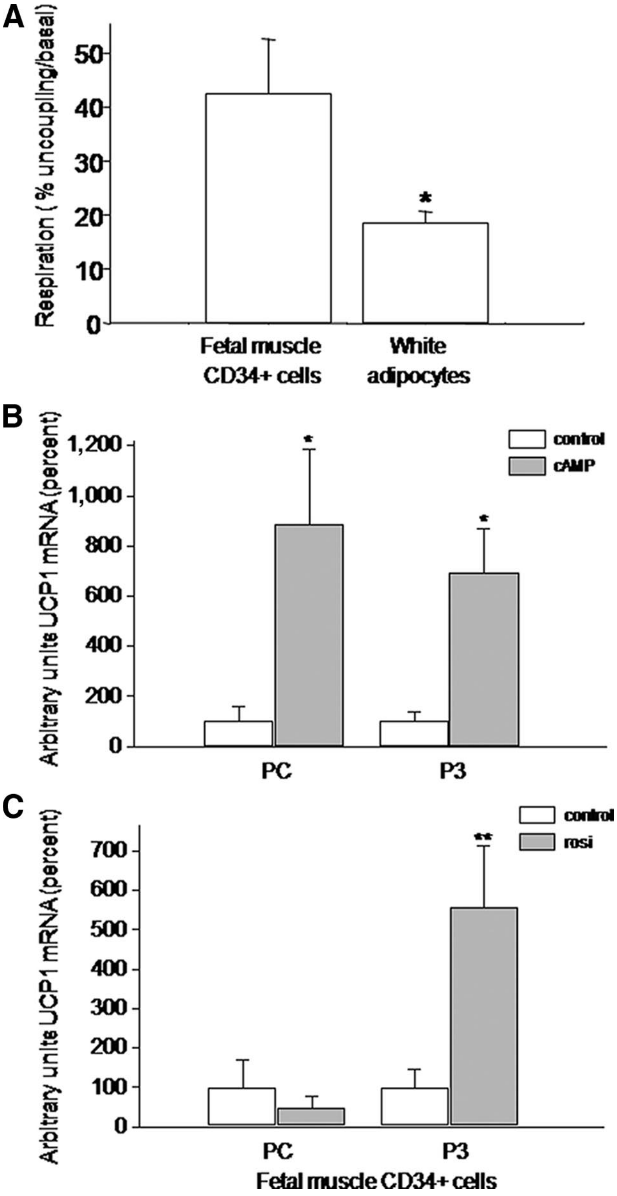

Figure 3. Uncoupling of mitochondrial respiration and control of UCP1 mRNA expression in human fetal muscle CD34+ cells. **(A):** Uncoupling of mitochondrial respiration in isolated fetal muscle CD34+ cells and in human adult white adipocytes grown in PC and freshly trypsinized. The results are means ± SEM. ∗, $p < .05$; $n = 3$. **(B):** Effects of the cAMP derivatives 8-bromo-cAMP (0.25 mM) and (4-chlorophenylthio)-cAMP (0.25 mM) (cAMP) on UCP1 mRNA expression in CD34+ cells in PC or expanded up to P3. All the cells were grown for 4–6 days in EGM2 medium and then placed for 8–12 days in the adipogenic medium described in Materials and Methods. The results are means ± SEM of arbitrary values normalized using the corresponding cyclophilin A values. Results are expressed in percentage of the respective untreated (control) values, considered as 100%. ∗, $p < .05$; $n = 3–6$. **(C):** Effects of 1 μM rosi on UCP1 mRNA expression in CD34+ cell PC or P3. The results are expressed as in **(B)**. ∗∗, $p < .01$; $n = 4–7$. Abbreviations: P3, passage 3; PC, primary culture; rosi, rosiglitazone; UCP1, uncoupling protein-1.

Muscle Specificity and Persistence Throughout Life of Human Brown Adipocyte Progenitors

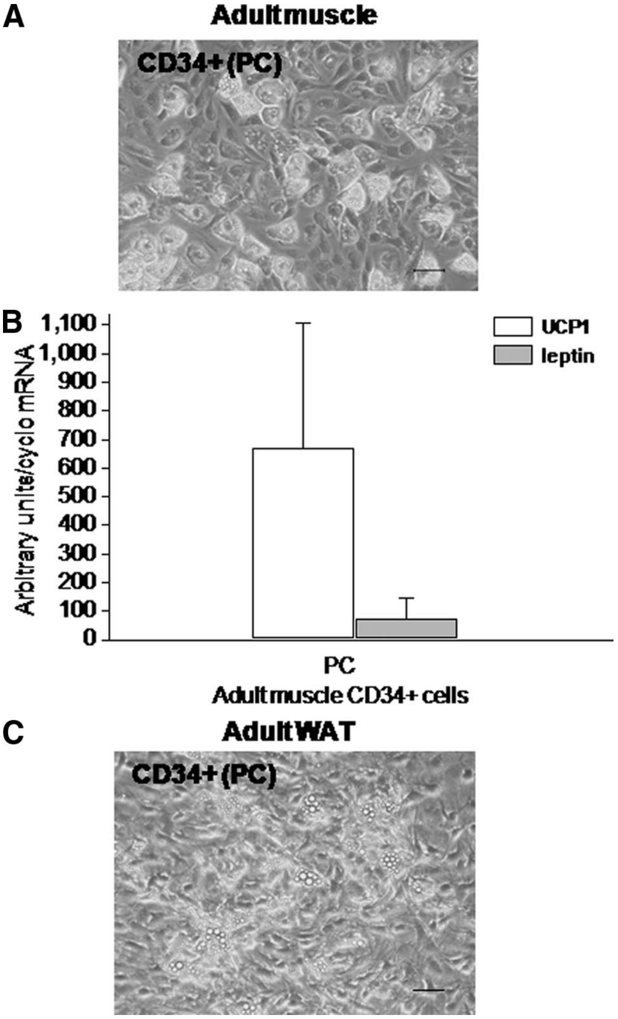

Figure 4. Characterization of adult muscle and WAT cells in adipogenic culture. **(A):** Human adult muscle CD34**+** cells in PC. Shown is a phase-contrast image. Scale bar = 50 μm. **(B):** Quantitative reverse transcription polymerase chain reaction determination of UCP1 (open column) and leptin (gray column) mRNA expression. All the cells were grown for 4–6 days in EGM2 medium and then placed for 8–12 days in the adipogenic medium described in Materials and Methods. The results are the mean ± SEM of arbitrary values normalized using the corresponding cyclophilin A values. $n = 4–5$. **(C):** Adult human WAT CD34+ cells in PC. Shown is a phase-contrast image. Scale bar = 50 μm. Abbreviations: PC, primary culture; UCP1, uncoupling protein-1; WAT, white adipose tissue.

The derivation of UCP1-expressing cells from human fetal muscle raised the question of the restriction of brown adipocyte progenitors to this tissue and to the fetal stage. To address this issue, CD34+ cells purified by FACS from human fetal pancreas, lung, and liver were cultured under the same adipogenic conditions as fetal muscle CD34+ cells. The sorted cells grew slowly, and only a small proportion of them became multilocular. UCP1 mRNA was not expressed in pancreas or lung cells; however, a minor expression was measured in liver cells, which amounted to 2% of that detected in fetal muscle CD34+ cells (not shown).

CD34+ cells sorted from four adult (50–78 years) human skeletal muscle samples, grown in primary culture under adipogenic conditions, also differentiated into multilocular cells. These cells were interspersed with other types of cells, some of them containing small lipid droplets (Fig. 4A). The level of UCP1 mRNA (370 ± 132 arbitrary units) was 21% of that detected in primary cultured fetal muscle CD34+ cells. In contrast, leptin expression (75 ± 69 arbitrary units) was 7.6-fold

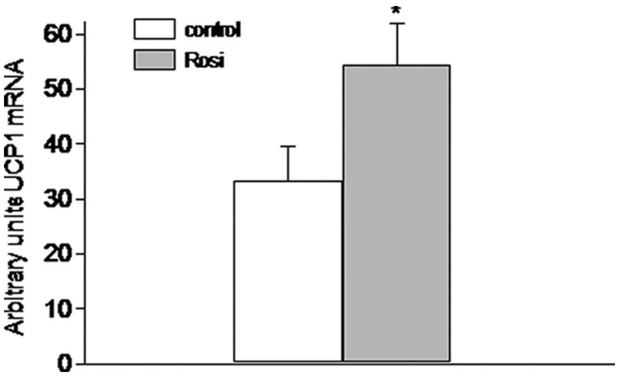

Figure 5. Effect of Rosi on UCP1 mRNA expression in human skeletal muscle. Quantitative reverse transcription (RT)-polymerase chain reaction (PCR) determination of UCP1 mRNA expression in the vastus lateralis of obese diabetic patients before and after treatment with Rosi (two doses of 4 mg each per day for 8 weeks). Since the RT-PCR conditions used were different, the arbitrary values of this figure cannot be compared with those of Figs. 2–4. The results are the means ± SEM of arbitrary values normalized using the corresponding cyclophilin A values. $n = 7$. *, $p < .05$ versus control. Abbreviations: Rosi, rosiglitazone; UCP1, uncoupling protein-1.

higher than in fetal cells (Fig. 4B). CD34+ cells sorted from four adult (45–55 years) human WAT samples were also grown in primary culture under adipogenic conditions. They became partially multilocular (Fig. 4C) but did not express UCP1 mRNA.

Detection of UCP1 mRNA Expression in Human Muscle and Effect of Rosiglitazone In Vivo

It was interesting to investigate the possibility that brown adipocyte progenitors of the adult human skeletal muscle can differentiate in vivo and give rise to UCP1-expressing cells. The presence of UCP1 mRNA in the adult human skeletal muscle was tracked using a high-sensitivity quantitative RT-PCR technique, and in fact, low levels of UCP1 mRNA were detected in the rectus abdominus muscle of 10 lean subjects (UCP1/cyclophilin A ratio, 24 ± 9; not shown). The PCR-amplified fragment was sequenced and found to be 100% homologous to human *UCP1* gene. The UCP1 mRNA level in adult human muscle was 75-fold lower than that in fetal muscle CD34+ cells in culture.

Since the PPARγ agonist rosiglitazone was a strong inducer of UCP1 mRNA expression in muscle CD34+ cells in culture, it seemed of interest to investigate the possible effect of this compound in vivo in humans. Vastus lateralis muscle biopsies from seven obese patients with type 2 diabetes mellitus treated for the management of their metabolic syndrome with rosiglitazone were used. The biopsies were obtained before and after 8 weeks of treatment with rosiglitazone (two doses of 4 mg each per day). As shown in Figure 5, the muscle UCP1 mRNA level was increased 1.6-fold by the roziglitazone treatment. Strong effects of rosiglitazone, varying between 1.5- and 4.1-fold, were observed in four of seven patients.

DISCUSSION

In the present study we demonstrated the existence, in human fetal and adult skeletal muscle, of a stationary population of cells expressing the CD34 surface protein and able to differentiate in vitro into genuine brown adipocytes expressing a high level of UCP1 mRNA. The potential of the CD34+ cells to differentiate into brown adipocytes was preserved throughout development and aging, although the UCP1/leptin ratio became smaller after expansion of the cells, suggesting a shift toward a white adipocyte phenotype. In immortalized HB2 brown adipocytes, UCP1 mRNA expression was found to diminish gradually upon repeated passages [37]. This loss and that observed in this study could be due to a negative growth advantage of cells with uncoupled respiration.

Measurements of mRNA transcripts by microarray in differentiated fetal muscle CD34+ cells first confirmed the expression of UCP1 mRNA previously evidenced by quantitative RT-PCR. It also suggested that these cells are well equipped in thermogenic transcription factors (PPARγ and PGC-1α) and, despite an adipocyte-like morphology, contain levels of mitochondria (COX IV and SDH) and of fatty acid degradation (CPT1B, ACAD, and ACADM) enzyme mRNAs that are of the same order of magnitude as those found in skeletal muscle. The lack of a detectable expression of myogenin, Myf5, and MyoD1 in differentiated CD34+ cells suggests that the adipogenic culture conditions used in this study are not permissive for muscle-specific gene expression. It is noteworthy that muscle CD34+ and, to a larger extent, CD146+ cells grown in a myogenic medium can differentiate into myocytes in vitro and in vivo [38]. The β_3-adrenoceptor, which, in rodents, is considered an adipocyte-specific β-adrenoceptor subtype [39], was expressed in the CD34+ cells, although at a low level. The presence of the β_3-adrenoceptor had been reported in humans only in newborn BAT [40] and in infant BAT immortalized cells [41]. Finally, Cidea mRNA expression confirmed the brown adipocyte nature of the CD34+ cells.

The part of the respiration that was uncoupled was higher in differentiated fetal muscle CD34+ cells than in adult white adipocytes. This observation suggests that UCP1 is functional and uncouples mitochondrial respiration in muscle-derived cells, behaving as a genuine uncoupling protein.

Cell-permeating cAMP derivatives and the PPARγ agonist rosiglitazone strongly stimulated UCP1 mRNA expression in expanded CD34+ cells, demonstrating the existence of cAMP signaling and the possibility of increasing UCP1 expression by specific β-adrenoceptor and PPARγ agonists in these cells. The observation that rosiglitazone had no effect on cells in primary culture is difficult to explain. It might be that the basal UCP1 mRNA expression, by decreasing upon cell expansion, becomes more susceptible to an upregulation by rosiglitazone.

Since in rodents, the presence of ectopic brown adipocytes in the WAT, as well as the possibility of increasing their number under conditions of high thermogenic needs such as cold acclimation, is well documented [5–7], we anticipated finding brown adipocyte progenitors in the adult human WAT. It should be mentioned that the absence of brown adipocyte progenitors in the human subcutaneous fat does not exclude the possibility that such progenitors could be found in other WAT depots in humans. In particular, it would be interesting to study the potential presence of brown adipocyte progenitors in the BAT-like depots detected by positron emission tomography in the upper part of the human body [14].

The unique and tissue-specific potentiality of human muscle CD34+ cells to transform into brown adipocytes is consistent with the observation that during normal development brown and white adipocytes derive from different precursor cells [42, 43] and with the recent demonstration that in rodent BAT brown adipocytes share a common origin with myocytes but not with white adipocytes [44]. Our results are in line with the recent finding of Almind et al. [15], who showed UCP1-positive brown fat cells in mouse intermuscular fat, but our study, using another approach, goes one step further since it allows the identification in the human skeletal muscle of the brown adipocyte progenitor cells. Previously, white preadipocytes were also characterized as CD34+ cells [42]. It has also been shown that pericytes can differentiate along the chondrocytic and adipocytic lineages in vitro [45]. It has been found that fetal muscle CD34−/CD146+/CD45−/CD56− cells in an adipogenic medium different from that used in the present study (i.e., consisting of DMEM containing 10% fetal calf serum, 1 μM dexamethasone, 0.5 μM isobutylmethylxanthine, 60 μM indomethacin, and 170 μM insulin) differentiated into multilocular cells but that the latter did not express UCP1 mRNA [38]. The CD146+ and CD34+ cells might therefore, under controlled culture conditions, differentiate into white and brown adipocytes, respectively. It is not known whether the brown adipocyte progenitors are the ancestral CD34+ cell or one particular endothelial CD34+ cell subtype [24]. Additional characterization and separation of the CD34+ cell pool would be necessary to answer this question.

In this study, the presence of a small but significant amount of UCP1 mRNA in the adult human skeletal muscle was demonstrated using quantitative RT-PCR. By analogy with the results obtained by Almind et al. [15] in mice, it can be assumed that the low level of UCP1 detected in adult human muscle is expressed by brown adipocytes infiltrating the muscle and not by the muscle fibers. The microarray approach did not allow detection of UCP1 in the adult human skeletal muscle, possibly because it is less sensitive than the quantitative RT-PCR used. Only one study, to our knowledge, has detected UCP1 mRNA in human skeletal muscle [46]. These findings suggest that part of the CD34+ progenitors can differentiate into brown adipocytes in the adult skeletal muscle, which opens the way for the possibility of actively recruiting brown adipocytes in human skeletal muscle via therapeutic targeting with potential antiobesity drugs.

Moreover, two arguments suggest a physiological relevance of our findings. First, our observation that cell-permeating cAMP derivatives strongly stimulate UCP1 mRNA expression in muscle CD34+ cells in culture has a corollary in the finding of Almind et al. [15], who showed that the administration of a β_3-adrenoceptor agonist for 7 days dramatically increased (32-fold) the UCP1 expression in obesity-resistant 129S6 mouse muscle. Second, rosiglitazone not only increased UCP1 expression in muscle CD34+ cells in long-term cultures but also stimulated UCP1 expression in adult human skeletal muscle in vivo. Thus, the pharmacological recruitment of CD34+ muscle progenitors into thermogenic cells in situ and, possibly, the stimulation of UCP1 expression in these cells by specific β-adrenoceptor or PPARγ agonists might reveal a new maneuver against obesity.

The identification of UCP1-expressing cell progenitors in human muscle might be of therapeutic significance and lead to the development of novel strategies against obesity. It was estimated that in the adult human, a 20%–25% increase in metabolic rate could be achieved by as little as 40–50 g of active BAT. Twenty percent of daily energy expenditure could make the difference between maintaining body weight and gaining body weight at a rate of 20 kg per year [47]. Furthermore, it has recently been shown that ectopic expression of very low levels of UCP1 in the mouse epidydimal fat reverses a high-fat diet-induced insulin and leptin resistance [48]. Consequently, autologous UCP1-expressing cells could be, after differentiation in vitro from muscle CD34+ progenitors, retransplanted into patients. However, the success of such a strategy depends on a perfect control of brown adipocyte differentiation, to avoid the emergence of white adipocytes from transplanted cells. Indeed, the muscle niche might be more adequate to sustain white than brown adipocyte differentiation, as revealed by the fatty infiltration observed in the muscle of patients with Duchenne muscular dystrophy [49].

CONCLUSION

All the studies concerning UCP1 expression in adult humans have until now been focused on the WAT. In the present study, we showed for the first time the existence in skeletal muscle and the prospective isolation of a reservoir of progenitor cells with a high potential for brown adipogenic differentiation and UCP1 expression at all stages of the development and adult life. This observation opens new avenues for fighting obesity by increasing heat dissipation.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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