Inducible Brown Adipogenesis of Supraclavicular Fat in Adult Humans

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Brown adipose tissue (BAT) plays key roles in thermogenesis and energy homeostasis in rodents. Metabolic imaging using positron emission tomography (PET)-computer tomography has identified significant depots of BAT in the supraclavicular fossa of adult humans. Whether supraclavicular fat contains precursor brown adipocytes is unknown. The aim of the present study was to determine the adipogenic potential of precursor cells in human supraclavicular fat. We obtained fat biopsies from the supraclavicular fossa of six individuals, as guided by PET-computer tomography, with paired sc fat biopsies as negative controls. Each piece of fat tissue was divided and processed for histology, gene analysis, and primary culture. Cells were examined for morphological changes in culture and harvested for RNA and protein upon full differentiation for analysis of *UCP1* level. Histological/molecular analysis of supraclavicular fat revealed higher abundance of BAT in PET-positive than PET-negative individuals. In all subjects, fibroblast-like cells isolated from supraclavicular fat differentiated *in vitro* and uniformly into adipocytes containing multilobulated lipid droplets, expressing high level of *UCP1*. The total duration required from inoculation to emergence of fibroblast-like cells was 32–34 and 40–42 d for PET-positive- and PET-negative-derived samples, respectively, whereas the time required to achieve full differentiation was 7 d, regardless of PET status. Precursor cells from sc fat failed to proliferate or express *UCP1*. In summary, preadipocytes isolated from supraclavicular fat are capable of differentiating into brown adipocytes *in vitro*, regardless of PET status. This study provides the first evidence of inducible brown adipogenesis in the supraclavicular region in adult humans. **(*Endocrinology* 152: 3597–3602, 2011)**

Brown adipose tissue (BAT) plays key roles in thermogenesis and energy homeostasis in rodents. It expresses a unique protein, uncoupling protein 1 (UCP1), which dissipates energy as heat (1).

Positron emission tomography (PET)-computer tomography (CT) has recently identified significant depots of BAT in adult humans (2), leading to a resurgence of interest in human BAT research (3). Initial investigations based on conventional single PET-CT studies reported a low prevalence of BAT in adult humans of 5–10% (4–6). Subsequent studies revealed conventional PET-CT to be insensitive and poorly reproducible for BAT detection (7, 8). Histological/molecular examination of supraclavicular fat has indicated a very high if not universal presence of BAT in adult humans (9). However, there is considerable variation in BAT abundance (7), with abundance up to 20 times lower in PET-negative (PET−ve) fat (9). What determines BAT abundance in human adults is unknown. It is possible that the status in adulthood is predetermined by residual remnants from infancy. Alternatively, it is possible that there is a dynamic regulation of precursor cell growth, differentiation, and apoptosis by factors yet to be identified. There is strong evidence from rodents for the existence of inducible brown adipogenesis. Whether the same is true in humans is unknown but clinically relevant because inducible brown adipogenesis could be exploited as a novel approach for the therapy of obesity.

BAT is present in supraclavicular but not in sc fat in adult humans (9). To test the hypothesis that supraclavic-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2011-1349 Received June 8, 2011. Accepted July 7, 2011.
First Published Online July 26, 2011

Abbreviations: BAT, Brown adipose tissue; CT, computer tomography; PET, positron emission tomography; PET+ve, PET positive; PET−ve, PET negative; SVF, stromal vascular fraction; UCP1, uncoupling protein 1.

ular fat harbors precursor brown adipocytes, we have undertaken *in vitro* studies to determine whether brown adipocytes can be induced in culture from stroma-vascular cells obtained from biopsy tissue.

Materials and Methods

Subjects and fat biopsies

Primary culture was established from fat biopsies obtained intraoperatively from the supraclavicular fossae of individuals undergoing head/neck surgery for malignancy. All patients underwent preoperative PET-CT. Primary culture established from paired biopsies of white adipose tissue obtained from anterior neck sc compartment of the same individuals served as negative controls (9).

PET-CT was performed at 20–22 C after a 6-h fast. Details of PET-CT scanning procedures and biopsy are described previously (9). Each piece of fat was divided into three pieces: the first two for histology/mRNA extraction and the third for primary culture. The presence of brown adipocytes was defined by identification of multilobulated lipid droplet and expression of UCP1 gene transcripts and/or protein. UCP1 expression was compared between biopsies and cultured cells.

RNA collection

RNA was extracted from whole adipose tissue/cultured adipocytes using Trizol and purified with ethanol precipitation on RNeasy minispin columns (QIAGEN, Doncaster, Australia). The average yield was 5–7 μg per 100 mg whole tissue and 8–10 μg for each 25-cm^2 flask of confluent primary adipocytes. RNA concentration/quality was assessed by a NanoDrop spectrophotometer (NanoDrop Technology, Wilmington, DE).

Quantitative RT-PCR

For quantification of target gene mRNA levels, 2 μg of RNA was reverse transcribed with a high-capacity cDNA kit (Applied Biosystems, Foster City, CA) in a total volume of 20 μl, as previously described (9). Standard TaqMan cycling conditions were used with TaqMan gene expression assays using the Applied Biosystems 7900HT (Applied Biosystems). All reactions were performed in triplicate. β-Actin was used as an internal standard. The relative expression of target mRNA was computed from the target cycle threshold values and the β-actin cycle threshold value using standard curve method (sequence detection systems chemistry guide; Applied Biosystems).

Immunohistochemistry

Adipose tissue biopsies were fixed in 10% formalin and molded in paraffin. Sections for immunohistochemistry were processed as previously described (9) and incubated with primary antibody [rabbit polyclonal to UCP1 (1:500); ab10983; Abcam, Cambridge, UK]. This was followed by 3 × 10-min washes in PBS and incubation with secondary antibody. Sections were sealed and imaged using Eclipse E800 microscope (Leica, Heerbrugg, Switzerland).

Western blot

Protein was extracted from homogenized whole tissue or primary adipocytes by incubation in lysis buffer (1 M Tris, pH 7.6; 5 M sodium chloride; 0.5 M EDTA; 1% Triton X-100; protease inhibitors) for 30 min at 4 C. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA) and 60 μg protein fractions were separated by 10% SDS-PAGE, blotted to a polyvinyl difluoride membrane (Immobilon P, Millipore), and detected by rabbit polyclonal antihuman-UCP1 (ab10983; Abcam).

Cell isolation and primary adipocyte culture

The method established in mice was modified for human BAT cultures, based on the identification that a mitotic compartment, termed the stromal vascular fraction (SVF), exists in adipose tissue (10, 11). Approximately 100 mg of adipose tissue was obtained from the supraclavicular fossa for the preparation of primary culture of brown adipocytes, as previously described (12). The cells were cultured in 25-cm^2 flask (Corning, Corning, NY) at a concentration of 0.6×10^6 cells/flask in 5 ml of medium. Although noradrenaline is the major regulator of brown adipogenesis in rodents, many other hormones and growth factors play key roles in BAT recruitment, proliferation, and differentiation, including thyroid hormone, peroxisomal proliferator-activated receptor-γ agonist, GH, and IGF-I, which induce brown adipogenesis *in vivo* and *in vitro* (1, 12–16). Based on known stimulants of brown adipogenesis in animals, a differentiating culture medium consisting of a β-agonist, a peroxisomal proliferator-activated receptor-γ agonist, IGF-I and various adipogenic factors was used.

The proliferation culture medium was DMEM with 10% (vol/vol) newborn calf serum (Invitrogen, Carlsbad, CA), 10 mM HEPES, 0.85 μM insulin, 1 nM triiodothyronine, 100 μM isoprenaline, 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were grown at 37 C in an atmosphere of 5% CO_2 in air with 80% humidity. The cells were washed in PBS and medium changed on d 1 and then every second day until they reached confluence. Cells were then maintained in differentiation culture medium supplemented with 10 μg/ml transferrin (Sigma, St. Louis, MO), 1 μM dexamethasone (Sigma), 500 μM isobutyl-methylxanthine (Sigma), 33 μM biotin (Sigma), 17 μM pantothenate (Sigma), 1 nM human GH, 1 nM IGF-I, and 1 μM rosiglitazone (Invitrogen). The cells were harvested for RNA isolation and Western blot when fully differentiated, as indicated by the presence of multilobulated lipid droplets.

The St. Vincent's Hospital Human Research Ethics Committee approved the study and subjects provided informed written consent.

Results

PET status and supraclavicular adipose tissue biopsy

Six patients were recruited and in whom PET-CT was positive in two and negative in four patients (Table 1).

TABLE 1. Patient characteristics

	PET+ve	PET−ve
n	2	4
Age (yr)	35–41	52–64
Gender	2F	1F:3 M
BMI (kg/m^2)	20–22	22–30
Diagnosis	Papillary thyroid cancer	Squamous cell carcinoma

BMI, Body mass index.

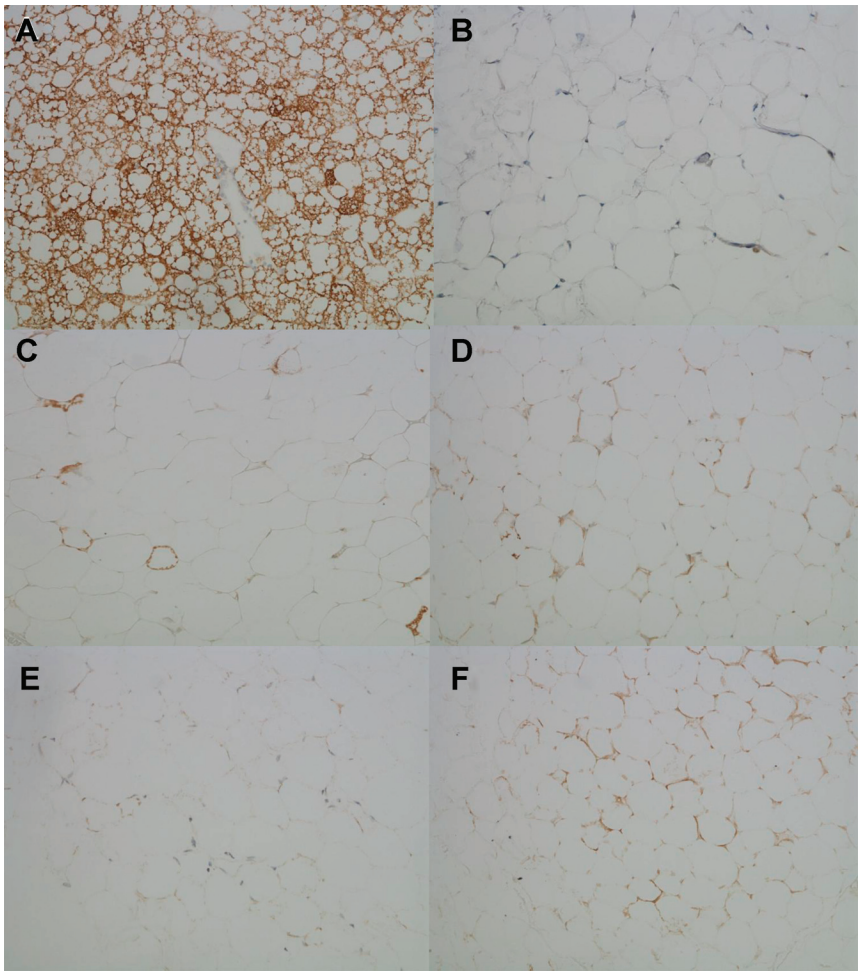

FIG. 1. Immunohistochemical features of supraclavicular and sc adipose tissue biopsies. Immunohistochemistry of PET+ve fat (n = 2) revealed uniform presence of adipocytes with multilobulated lipid droplets, strongly reactive for UCP1 (A: UCP1 immunohistochemistry, ×20). Subcutaneous fat showed only adipocytes with unilobulated lipid droplets with no UCP1 staining (B: UCP1 immunohistochemistry, ×20). In contrast, PET−ve fat in one patient consisted primarily of adipocytes with unilobulated lipid droplets, devoid of UCP1 staining. There were scattered adipocytes with multilobulated lipid droplets, which showed UCP1 staining (C: UCP1 immunohistochemistry, ×20). PET−ve fat from the other three patients showed more irregular scattering of UCP1-positive adipocytes of varying intensity (D–F: UCP1 immunohistochemistry, ×20).

Among PET positive (PET+ve) individuals, *UCP1* expression was more than 4 orders of magnitude higher in supraclavicular compared with sc fat. Histology showed uniform presence of adipocytes with multilobulated lipid droplets, typical of brown adipocytes. These cells stained strongly for UCP1, confirming the presence of BAT (Fig. 1A).

Among PET−ve individuals, *UCP1* expression was 7.7 times greater (95% confidence interval 4.2–12.6; $P < 0.01$) in supraclavicular compared with sc fat. Histology in one patient revealed a predominance of adipocytes with unilobulated lipid droplets, with an occasional scattering of UCP1-positive cells displaying multilobulated lipid droplets (Fig. 1C), characteristic of brown adipocytes. More irregular scattering of UCP1-positve cells of different intensity was observed in the remaining three patients (Fig. 1, D–F).

Culture of adipose precursor cells

Primary cultures were established from biopsies obtained from the supraclavicular fossae in all six patients (Fig. 2, A–F).

Proliferation of preadipocytes

After 2 d in culture, adherent cells isolated from the SVF were spherical and present singly or in pairs, characteristic of undifferentiated preadipocytes (Fig. 2A). The cells were examined daily. During the first 30 d of culture, no morphological differences of preadipocytes were observed. Inoculation density (0.5–1.5 × 10^6 cells/flask) and isoprenaline concentrations (50–200 μM) did not affect the confluence/morphology of preadipocytes.

Fibroblast-like cells emerged among undifferentiated cells in clusters after 30 d in culture (Fig. 2C), characterized by an elongated fusiform morphology with a single nucleus and abundant cytoplasm. Their cell membrane displayed a coat of coarsely granular material with no cytoplasmic lipid droplets. Once these cells appeared, remaining cells transformed into fibroblast-like cells within 1–2 d, and flasks reached more than 80% confluence within a week (Fig. 2D).

Differentiation of confluent preadipocytes

On exposure to differentiation medium, cells rapidly became polygonal and accumulated lipid within cytoplasm. Small lipid droplets were visible in a ring around the central nucleus by d 2. As fat accumulation continued, the lipid content of each cell remained dispersed in small fat droplets. By d 7, fully differentiated cells denoted by the accumulation of multilobulated lipid droplets were observed (Fig. 2, E and F).

Differences between cells in relation to PET status

We compared the proliferation/differentiation pace of cells derived from PET+ve and PET−ve fat. The time taken for fibroblast-like cells to emerge was 32–34 and 40–42 d, and the duration to reach confluence was 5 and 7 d, for PET+ve and PET−ve derived fat biopsies, respec-

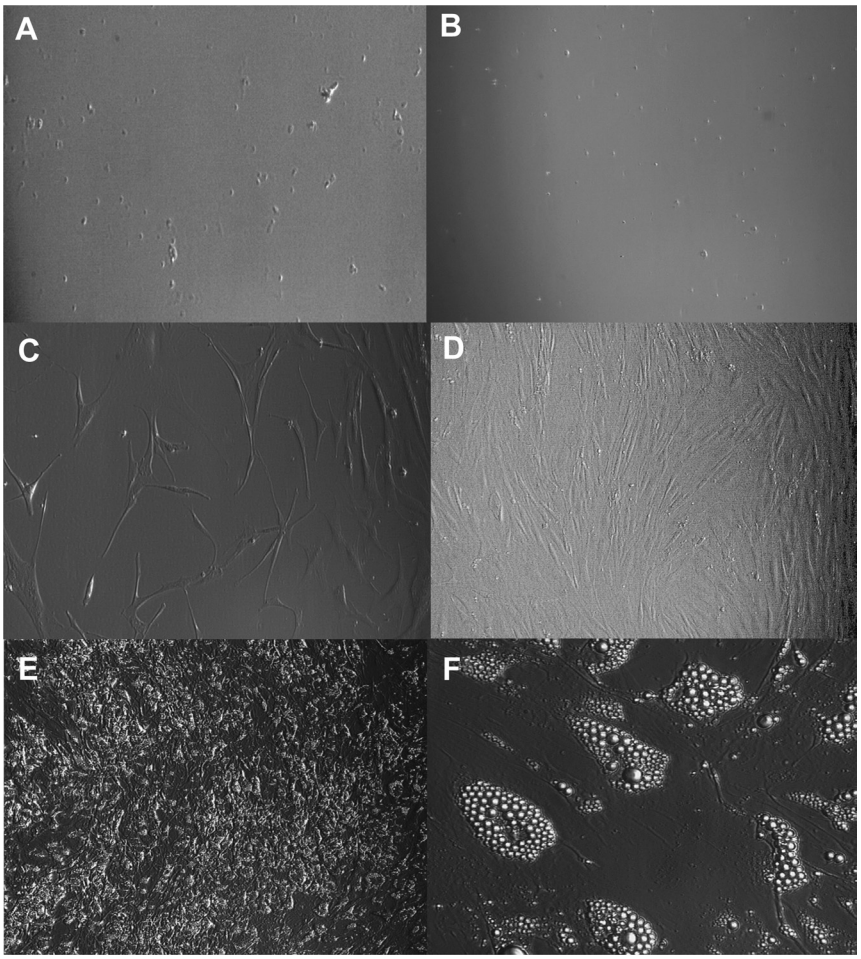

FIG. 2. Cytological features of primary adipocytes from supraclavicular and sc fat. Precursor cells isolated from the stroma-vascular fraction of supraclavicular (A: d 2, ×20x) and sc fat (Panel B: d 2, ×20) were single and spherical with no discernable morphological differences. Only precursor cells from supraclavicular fat showed progressive proliferation to fibroblast-like cells (C: d 30–32, ×20), which reached confluence after a further 5–7 d (D: ×20). These cells were devoid of any lipid droplets. Cells began lipid droplet accumulation from d 2 after incubation in differentiation medium. Fully differentiated brown adipocytes displayed multilobulated lipid droplets by d 7 (E: ×10 and F: ×40) expressing high levels of UCP1 (Fig. 3).

tively. The duration required for fibroblast-like cells to fully differentiate was 7 d, regardless of PET status.

There were no discernable morphological differences between undifferentiated, fibroblast-like, or final fully differentiated cells from PET+ve and PET−ve fat biopsies.

Subcutaneous fat-negative control

Subcutaneous fat biopsies displayed adipocytes with unilobulated lipid droplets with absent UCP1 staining (Fig. 1B). Cells isolated from sc fat displayed similar morphological features as those isolated from supraclavicular fat (Fig. 2B). Cultures were undertaken in all subjects in parallel with those isolated from corresponding supraclavicular fat. Prolonged incubation for up to 7 wk in proliferative medium failed to induce confluence or the development of fibroblast-like cells (data not shown). The evaluation of the cultures was terminated beyond 7 wk.

UCP1 expression

Gene and protein expression of UCP1 was quantified in primary culture established from supraclavicular and sc fat (Fig. 3, A and B). UCP1 mRNA and protein were detected in fully differentiated cells from supraclavicular but not from those cultured from sc fat. The abundance of UCP1 was similar between PET+ve and PET−ve fat.

Discussion

This study demonstrates the successful culture of adipose precursor cells from BAT in adult humans. Under defined conditions, preadipocytes isolated from supraclavicular adipose tissue differentiate into brown adipocytes, sharing similar morphological/molecular characteristics as mature brown adipocytes. Primary culture of precursors established from PET−ve fat biopsies required a longer incubation period to reach confluence. However, the final phenotype of brown adipocytes was identical for precursor cells obtained from PET+ve and PET−ve fat.

In rodents, an incubation time of 7–10 d is required to establish primary brown adipocyte in culture (10). By contrast, an incubation period beyond 50 d was required for precursor cells to fully differentiate into brown adipocytes in humans. The reason for the big difference in duration is unknown. It could be due to the differences in biogenic potential between species or the relative paucity in precursor cell abundance in humans. It is also probable that the optimal conditions for human cells have not yet been defined, given that the conditions used were adopted from the rodent literature. The apparently long incubation required for proliferation and differentiation may explain the paucity of primary brown adipocyte cultures as an experimental model for study. The only successful establishment of primary culture of human brown adipocytes was derived from neonatal perinephric fat (17). Characterization was based on morphological identification of differentiated adipocytes cells harboring multilobulated lipid droplets. The authors did not quantify UCP1 expression for confirmation of brown adipocyte identity. Others

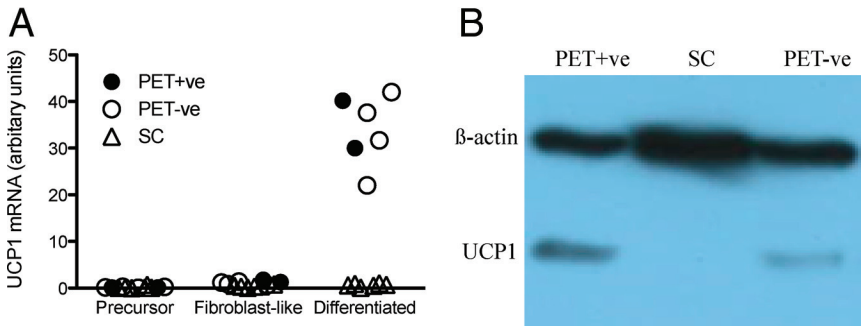

FIG. 3. Molecular features of primary adipocytes from supraclavicular and sc fat. *UCP1* expression (A) was hardly detectible in precursor cells and fibroblast-like cells derived from PET+ve (n = 2) and PET−ve fat (n = 4). Fully differentiated cells displayed similar *UCP1* level, regardless of PET status. *UCP1* was not detected in cultured cells derived from sc fat (n = 6) harvested at identical analysis times of corresponding cultured cells from supraclavicular fat. Western blot showed levels of UCP1 in differentiated adipocytes from primary culture established from PET+ve and PET−ve fat (B). Subcutaneous fat was used as negative control and β-actin as a protein loading control. Quantitative RT-PCR was performed in triplicates.

have established cultures from adenovirus-mediated expression of master regulators of adipogenesis (*e.g.* peroxisomal proliferator-activated receptor-γ coactivator-1α), with stem cells or immortalized cell lines. However, the appropriateness of these models for regulatory studies is uncertain (13, 18). At present, studies investigating the factors regulating human BAT activity *in vivo* are limited by high radiation exposure of PET-CT. The successful development of primary brown adipocytes in culture provides a promising model for *in vitro* studies of the humoral/endocrine regulation of human BAT.

The present findings provide clues on the developmental origin of human brown adipocytes. The primary cultures of brown adipocytes were derived from undifferentiated precursor cells of the stroma and not from preexisting mature BAT *in situ*. The cultures were successfully established from not only BAT abundant PET+ve fat but also PET−ve fat in which mature brown adipocyte population is scant. SVF cells from sc fat failed to differentiate into mature brown adipocytes under identical culture conditions, suggesting that the developmental origins of supraclavicular and sc fat are different. A unique population of precursor cells with full brown adipogenic potential have been identified in animals and in human skeletal muscle. These mesenchymal progenitor cells express adipomyogenic markers such as *BMP7* and *PRDM16* (19, 20) and *CD34* (21), respectively. The limited availability of human supraclavicular adipose tissue in the current study unfortunately precluded more detailed gene profiling of cultured cells to evaluate their developmental origin.

There are several issues to consider, given our experimental design. Primary culture was established from biopsies obtained from patients with malignancies in whom indications for PET-CT imaging offered the opportunity to correlate metabolic function to histology and required surgery allow access to the supraclavicular fossa. This resulted in inevitable heterogeneity in patient characteristics, such as age, gender, and body mass index. However, the success in establishing primary culture from all six patients provides strong evidence that BAT arises from precursor cells. The presence of BAT in the adult is unlikely to represent preexisting remnants from infancy. It is unlikely that underlying malignancy is a factor because we did not observe an association between active malignancy and the presence BAT in our previous investigation in more than 3000 patients with a history of malignant disease (7).

Our findings have important clinical implications. The inducibility *in vitro* to mature brown adipocytes suggests the potential for activation of indolent precursor cells *in vivo*. For example, the conversion from negative to positive PET status such as by cold simulation is likely mediated by changes in the neuroendocrine milieu of the supraclavicular microenvironment (22). Although it is likely thermogenic stimulation represents activation of preexisting brown adipocytes, it is equally likely that the microenvironment may also regulate the proliferation and differentiation of precommitted preadipocytes. Cold acclimatization stimulates the recruitment, proliferation, and differentiation of BAT in rodents (23), implying chronic cold exposure could be a physiologic switch to turn on BAT in humans, as supported by the observation of abundant BAT in outdoor workers exposed to cold (24). The factors that drive human BAT development and activity is poorly understood; however, the availability of primary adipocytes in culture presents a model for investigating key regulatory factors involved.

In summary, the morphological and molecular findings presented in the current study indicate that precursor cells isolated from supraclavicular adipose tissue proliferate and differentiate into mature brown adipocytes. This study provides the first evidence for inducible brown adipogenesis in adult humans. The establishment of primary cultures of BAT offers potential for use as a model to identify factors regulating their proliferation, differentiation and function, which may provide information that could lead to pharmacological activation of BAT for therapeutic purposes.

Acknowledgments

We thank Professor Barbara Cannon for helpful discussion during the conduct of this work.

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This work was supported by the National Health Medical Research Council (NHMRC) of Australia and by funding from St. Vincent's Clinic Foundation. P.L. was supported by a postgraduate research scholarship of the NHMRC and the Diabetes Australia Fellowship. M.M.S. is supported by an NHMRC Program Grant, "Pathways to Diabetes Prevention."

Disclosure Summary: None of the authors have any conflicts of interest to disclose.

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