Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat

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The imbalance between energy intake and expenditure is the underlying cause of the current obesity and diabetes pandemics. Central to these pathologies is the fat depot: white adipose tissue (WAT) stores excess calories, and brown adipose tissue (BAT) consumes fuel for thermogenesis using tissue-specific uncoupling protein 1 (UCP1)1–2. BAT was once thought to have a functional role in rodents and human infants only, but it has been recently shown that in response to mild cold exposure, adult human BAT consumes more glucose per gram than any other tissue3. In addition to this nonshivering thermogenesis, human BAT may also combat weight gain by becoming more active in the setting of increased whole-body energy intake4–7. This phenomenon of BAT-mediated diet-induced thermogenesis has been observed in rodents8 and suggests that activation of human BAT could be used as a safe treatment for obesity and metabolic dysregulation9. In this study, we isolated anatomically defined neck fat from adult human volunteers and compared its gene expression, differentiation capacity and basal oxygen consumption to different mouse adipose depots. Although the properties of human neck fat vary substantially between individuals, some human samples share many similarities with classical, also called constitutive, rodent BAT.

Initial examinations of principal human neck fat depots revealed brown adipocytes with classic multilocular adipocytes, numerous mitochondria and a rich investment with capillaries and sympathetic neurons10, as well as high expression of UCP1 (refs 10,11).

The microanatomical distinctions persisted at the level of gene expression. Nonparametric analysis of variance demonstrated a significant difference among the depots for expression of WAT-associated leptin gene, LEP, which was more enriched in superficial fat (P = 0.002) (Fig. 1c and Supplementary Table 3). We found the opposite for UCP1 expression (P = 0.002). Specifically, in two of the deeper fat depots, carotid sheath and longus colli, UCP1 expression was 12- to 72-fold higher than in the two superficial depots, subcutaneous and subplatysmal (P = 0.03 or lower for all four pairwise comparisons). The prevertebral site also had higher UCP1 expression than subcutaneous fat (P = 0.006). Therefore, although there was great variability among adult human neck fat in terms of UCP1 expression, we consistently found BAT to be most abundant near the carotid sheath and longus colli muscles. These deep locations may have functional importance: the longus colli depot is adjacent to the sympathetic chain, which mediates the rapid neuronal response to cold13, and the carotid sheath BAT envelops the carotid arteries, permitting effective heating of the cerebral vasculature14.

Having established the anatomical location of human neck BAT, we determined its probable developmental lineage. It has recently been shown using mouse tissue that brown adipocytes are not all the same. The large and constitutively present interscapular BAT (iBAT) derives from a lineage common to skeletal muscle and is termed classical or constitutive BAT (called here classical/constitutive BAT)15–17, whereas other brown adipocytes can be induced to grow within predominantly white depots and have been termed beige, brite or inducible BAT (called here beige/brite BAT)16–19. To provide more...
The three complementary marker analyses indicate that deep human neck brown adipocytes most closely resemble cells from the classical/constitutive BAT lineage in the mouse. This designation is supported by anatomical studies showing that this depot is present in human infants and persists even into the eighth decade of life. Functionally, this may also indicate that human neck BAT may have the same capacity for high rates of energy expenditure seen in rodent iBAT. Of note, these results represent the combined signal from whole-tissue biopsies and not individual cells. Given the mixed composition of human BAT, our findings are also consistent with the presence of some beige/brite adipocytes in the neck depot, as the beige marker TNFRSF9 (ref. 19) showed a nonsignificant, twofold enrichment in the deeper tissue and clustered the closest to UCP1 after the brown markers ZIC1 and LHX8. That some genes previously designated as markers of a brown adipocyte lineage in mice (EBF3, MPZL2 and FBXO31) did not have higher expression in the deeper human neck depot compared to the superficial depots or associate closely with UCP1 reflects the possibility that adipose tissue actually comprises multiple miniorgans both in mice and humans, with distinct developmental and functional characteristics. A clear definition of what these cell types are, their origins and their gene signatures is still a work in progress.

To determine whether adult human neck BAT also has a functional profile similar to that of classical/constitutive rodent BAT, we compared the expression of genes associated with rodent WAT and BAT differentiation, function and thermogenesis. We measured
gene expression in five different depots from five mice each: iBAT, inguinal subcutaneous WAT, perigonadal WAT, mesenteric WAT and perirenal WAT. We analyzed these five depots together with the deep neck fat from three individuals with high UCP1 expression, meaning the fat from these individuals probably had the highest proportion of brown adipocytes. We used the associated WAT from the subcutaneous neck depots of these individuals for comparison. Human BAT had an expression pattern that was very similar to that of mouse iBAT (Supplementary Table 3 and Figure 2a), including high expression of genes involved with mitochondrial biogenesis and thermogenesis (UCP1, PPARG1A, also known as PGC1α, and DIO2) and low expression of NRIP1, also known as RIP140, a nuclear co-repressor whose lowered expression leads to higher oxidative metabolism and mitochondrial biogenesis. Human subcutaneous WAT was strikingly different and was more similar to mouse mesenteric and perigonadal depots.

The ability to grow new functional brown adipocytes is probably essential for using BAT thermogenesis to treat metabolic dysregulation. We isolated preadipocytes from the stromal vascular fraction (SVF) of neck adipose tissue and differentiated them with cAMP with significantly higher expression of several BAT genes, including UCP1 (P = 0.04) and PPARG1C1 (P = 0.01) (Fig. 3b and Supplementary Table 6). These higher levels of gene expression demonstrate that as with the SVF from supraclavicular sites, the in vitro–differentiated cells from neck fat have the capacity to respond to adrenergic stimulation with expression of genes needed for thermogenesis and are therefore bona fide brown adipocytes. That the expression of PRDM16 was not as high in response to stimulation compared
Figure 3 Adult human neck BAT expresses functional genes similarly to mouse iBAT, can be grown ex vivo from multiple adipose tissue depots and has an unstimulated energy expenditure similar to that of mouse iBAT. (a) Gene expression of BAT- and WAT-associated genes measured in five different mouse adipose tissue depots taken from five 12-week-old male C57BL/6 mice. The genes from the mouse depots were clustered with the results from the samples of human deep neck fat that had the highest expression of UCP1, along with the associated subcutaneous fat samples. The heat map represents the relative abundance of each gene across all 31 samples. The values were normalized within each row using a linear color scale. The highest value in each row is bright red, the lowest is bright blue, and values near the midpoint between the highest and lowest are black. Missing values are gray. (b) The expression (shown as a fold change compared to vehicle treatment of the same duration) of four BAT-associated genes, UCP1, PPARGC1A, DIO2 and PRDM16, in preadipocytes from the SVF of both superficial and deep neck adipose tissue fat depots from four individuals (eight different sites in total) that were differentiated into mature adipocytes and then treated for 4 h with 500 µM dibutyryl-cAMP (DB-cAMP). *P < 0.05 determined by paired t test. Errors bars, s.e.m. (c) Basal OCR in adipose tissue from mouse iBAT or human cervical adipose tissue from subcutaneous (SQ) and longus colli (LC) depots from four individuals. The prefixes m and h indicated mouse and human, respectively. Note that we were unable to obtain tissue from the longus colli depot of patient 3, and so results from this patient are not shown. *P < 0.05 determined by unpaired t test. Errors bars, s.e.m.

Methods and any associated references are available in the online version of the paper.

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Author Contributions

A.M.C., A.P.W., R.X. and Y.-H.T. designed the experiments. A.M.C., A.P.W., C.V., T.J.S., C.A.S. and Y.-H.T. wrote the manuscript. T.L.H. did immunohistochemistry. A.M.C., A.P.W., R.X., C.A.S., C.R.-T., L.S.W., C.S., A.T.C., L.N.D., L.M.H., N.T., A.L.G., A.R.H., A.G., P.-O.H., M.A.M. and M.M. did the human gene expression profiling. C.A.S., N.T. and M.A.M. did the mouse gene expression profiling. T.J.S. performed brown adipogenesis experiments. C.V. and C.A.S. performed bioenergetic experiments. All authors contributed to editing the manuscript.

Competing Financial Interests

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Human study population. This study followed the institutional guidelines of and was approved by the Human Studies Institutional Review Boards of Beth Israel Deaconess Medical Center, Joslin Diabetes Center and Massachusetts General Hospital. Individuals were identified either by A.P.W. before anterior cervical spine surgery or P.-O.H. before thyroidectomy, and written informed consent was obtained by other study staff before surgery. All people undergoing thyroidectomies had thyroid-stimulating hormone values within the normal range. There were two independent cohorts: for anatomical localization, we performed light microscopy and compared to mouse adipose tissue depots, neck fat from 18 individuals was studied, and for lineage tracing, neck fat from 13 different people was studied. Healthy volunteers for MRI were recruited through electronic advertisements.

MRI. Studies were carried out using a 3.0T Siemens Allegra MRI System equipped for echo planar imaging with a quadrature head coil. The adult human volunteer lay supine in the scanner with the head immobilized using cushioned supports. Two sets of structural images were collected using a T1-weighted MPRAGE sequence (TR/TE = 2.73 ms/3.19 ms, flip angle = 7°, field of view (FOV) = 256 mm × 256 mm, slice thickness = 1.33 mm).

Light and electron microscopy. For light microscopy, we placed freshly resected brown and white fat into 4% PBS-buffered formalin (Sigma) and processed it as described32. Immunohistochemical assays were done with the use of polyclonal goat IgG to UCP1 (Santa Cruz Biotechnology, sc6258) at 1:50 dilution. For light and transmission electron microscopy, fat was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, osmicated and embedded in plastic (Araldite) and then processed as described in the Joslin Advanced Microscopy Core33.

Gene expression for anatomical localization of adult human neck fat. Freshly resected fat from the neck was placed immediately into RNA later (Qiagen). We extracted total cellular RNA from tissue using an RNeasy minikit (Qiagen) according to the manufacturer’s instructions. Quantity and purity were assessed by ultraviolet absorbance at 260 nm and 280 nm. Complementary DNA (cDNA) was prepared from 6 ng/µl of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Six microliters (36 ng) of cDNA were used in a 20-µL PCR using TaqMan Gene Expression Assays with a FAM dye label for the following genes (Supplementary Tables 3 and 5): UCP1, Dio2 (type 2 deiodinase), ADRB3 (β3-adrenergic receptor), PPARG1A (peroxisome proliferator-activated receptor γ coactivator-1α), CIDEA (cell death–inducing DNA fragmentation factor α-like effector A), PRDM16 (PRD1-BF1-RIZ1 homologous domain containing 16), NRIP1 (receptor-interacting protein 140), FBNI (fibulin 1), EN1 (engrailed 1), HOXA5 (homeobox A5), HOXC9 (homeobox C9) and LEP (leptin). Quantitative RT-PCR assays were run in duplicates and quantified in the ABI Prism 7900 sequence-detection system. The values were normalized to the expression of TBP (TATA-binding protein) in each sample, and results were expressed as ratios in arbitrary units.

Gene expression and cluster analysis of markers of adipocyte lineage. From 13 individuals undergoing routine neck surgery, we resected fat and prepared cDNA as described above using primer sequences for quantitative real-time PCR (Supplementary Table 4). Assays were run in duplicates and quantified in the ABI Prism 7900 sequence-detection system using SYBR Green as previously described32 for the following genes: TNFRSF9 (tumor necrosis factor receptor superfamily, member 9), EBF3 (early B cell factor 3), MPZL2 (myelin protein zero-like 2), FBXO31 (F-box protein 31), LHXB (LIM homebox 8), SHOX2 (short stature homeobox 2), TBX1 (T-box 1), TMEM26 (transmembrane protein 26) and ZIC1 (Zic family member 1). We normalized values to the expression of 18S ribosomal RNA in each sample and expressed results as ratios in arbitrary units. WAT was defined as the subcutaneous fat, and BAT was defined as the sample of deep fat that had the highest expression of UCP1. We clustered genes using pairwise complete-linkage hierarchical clustering according to the city-block distance between rows. The clustering visualization was done using GenePattern34.

Heat map for mouse-human comparisons. All mice were housed and used according to the institutional guidelines stipulated by the Joslin Diabetes Center Institutional Animal Care and Usage Committee. We resected mouse fat depots from 12-week-old male C57BL/6 mice (Taconic). Human and mouse RNA were isolated and measured as described above. After normalization to TBP, the gene expressions were log transformed. We generated the heat map with GenePattern34 using the relative abundance of each gene across all 31 samples.

Ex vivo differentiation. For isolation of preadipocytes, we isolated the SVF from superficial fat (pooled subcutaneous and subplatsymal) and fat located in the deeper neck regions (pooled carotid sheath, longus colli and prevertebral). Freshly resected fat from the neck was collected, minced and digested using collagenase 1 (2 mg/ml in PBS with the addition of 3.5% BSA; Worthington Biochemical Corporation), and the SVF was isolated as previously described35. SVF cells were plated and grown until 90% confluence in growth medium supplemented with 10% FBS in the presence of 5 ng/ml human basic fibroblast growth factor (Sigma–Aldrich), 10 ng/ml human epidermal growth factor, 10 ng/ml platelet-derived growth factor BB (both from PeproTech) and 10 ng/ml murine leukemia inhibitory factor (EMD Millipore)36,37. We seeded cells at 15,000 cells per well of a 48-well plate and grew them for 2 d until confluent. They were then differentiated for 10 d in growth medium without growth factors and supplemented with 2% FBS and adipogenic induction cocktail (50 µM indomethacin, 0.5 µM insulin, 33 µM biotin, 17 µM panthothenate, 0.1 µM dexamethasone, 2 nM I/30 and 540 µM isobutyryl-cAMP or vehicle for 4 h and then isolated RNA as described32.

Bioenergetics. Studies were carried out as described previously37. We collected adipose tissue samples from individuals during surgery and from 12- to 14-week-old male C57BL/6 mice. Both sets of tissues were rinsed with unbuffered KHB medium containing 111 mM NaCl, 4.7 mM KCl, 2 mM MgSO4, 1.2 mM Na2HPO4, 0.5 mM carnitine, 2.5 mM glucose and 10 mM sodium pyruvate, cut into pieces (~10 mg) and washed extensively, and then each piece was placed in a single well of a XF24-well Islet Flux plate (#101174-100, Seahorse Bioscience) and covered with a customized screen that allows for free perfusion while minimizing tissue movement. We added KHB (450 µl) to each well and studied the samples in an XF24 extracellular flux analyzer machine using the following protocol: oxygen concentration was measured over time periods of 2 min at 6-min intervals, consisting of a 2-min mixing period and a 4-min waiting period. Basal OCR was measured for each sample in triplicate. Each reported OCR value was an average of five independent pieces per tissue per experiment for mice and two to three independent pieces for the individuals.

Statistical analyses. We analyzed the data using JMP Pro 9.0.2 software (SAS Institute, Inc., Cary, NC). All P values presented are two tailed, and P < 0.05 was considered to indicate statistical significance. Comparison of the gene expression in different anatomical depots was done using the nonparametric Wilcoxon sign-ranks test. We generated principal components using log-transformed gene expression values by the principal components method with diagonals of 1 and did orthogonal rotation using the varimax method. Values are factor loadings, the correlation coefficient of the relationship between the components produced and the individual adipose tissue genes.

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