Brown and white adipose tissues: intrinsic differences in gene expression and response to cold exposure in mice

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Submitted 29 August 2013; accepted in final form 14 February 2014

Brown and white adipose tissues: intrinsic differences in gene expression and response to cold exposure in mice. Am J Physiol Endocrinol Metab 306: E945–E964, 2014. First published February 18, 2014; doi:10.1152/ajpendo.00473.2013.—Brown adipocytes (BAT) store energy in the form of triacylglycerol (TAG) and dissipate energy as heat, whereas white adipocytes (WAT) store energy in the form of triacylglycerol and glucose uptake and oxidation (9). WAT and BAT have significant transcriptional, secretory, morphological, and metabolic differences. The main murine BAT depot is located subcutaneously in the interscapular region, but it can also be found in the cervical, axillary, and paraaortic regions. BAT is a highly vascularized and innervated tissue, and brown adipocytes possess multilocular lipid droplets and a high number of mitochondria and demonstrate a high rate of fatty acid oxidation (9). WAT is found primarily under the skin (subcutaneous) or inside the abdomen (gonadal, mesenteric, omental, and perirenal), and it is much less innervated and vascularized than BAT. White adipocytes have a unilocular lipid droplet, few mitochondria, and a low oxidative rate. Important differences exist between subcutaneous and visceral depots, which support their different involvement in insulin sensitivity and type 2 diabetes, with visceral WAT being more closely associated with an adverse metabolic profile (28).

In recent years, considerable interest has risen in the potential beneficial effects of acquiring BAT features (browning) in nonclassic BAT locations such as WAT. It is known that subcutaneous BAT is more sensitive to acquisition of BAT characteristics than visceral depots in mice and humans (47, 53). However, it is still a matter of debate as to whether mature white adipocytes undergo transdifferentiation into brown adipocytes (12, 16) or whether precursors committed to the brown lineage already present in the tissue differentiate into mature brown adipocytes (42, 63). Brown in white (brite) or “beige” adipocytes are distinct from the adipocytes in interscapular BAT in that they arise as multilocular adipocytes in WAT during adrenergic activation (30, 42, 46, 61, 63). They have low levels of uncoupling protein 1 (UCP1) compared with classic brown adipocytes but possess the capacity to induce UCP1 expression to a very high extent and the ability to increase their oxygen consumption upon adrenergic activation. It has been reported recently that the adipocytes found in human BAT...
could be of this brite type, and a defined bona fide interscapular BAT depot is found in infants (13, 31, 36, 49, 63). Several classes of factors have been identified to promote a BAT phenotype either in white adipocytes and/or WAT. These include transcription factors [e.g., peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer-binding protein beta (C/EBPβ), and Foxc2], coregulators [e.g., PPARγ coactivator-1α (PGC-1α), Prdm16, and PGC1α], growth factors [e.g., bone morphogenetic protein (Bmp7), Fgf21, Bdnf, and Bmp8b], enzymes (e.g., Ptg2), lipid droplet-associated proteins (e.g., perilipin), and cell survival factors (e.g., neclin) as well as the miR193b-365 microRNA cluster (reviewed in Ref. 51). Although these factors have important roles in promoting a brown fat phenotype, to date, only fibroblast growth factor 21 (FGF21) has been reported in a therapeutic study (17). Understanding WAT-BAT plasticity in different depots, it is important for the design of future treatments to target type 2 diabetes and obesity by BAT-inducing drugs or other therapies, such as tissue transplantation or gene therapy.

For this study, we have compared gene expression in four different mouse adipose depots with very specific physiologies upon prolonged cold exposure. We sought to identify differentially regulated genes and thereby novel signaling pathways during cold-induced adipose tissue “remodeling.” Furthermore, we identified motifs that are enriched in the promoters of brite genes. We have been able to confirm that subcutaneous adipose tissue is the most prone to changing to display a brite phenotype identified as a number of temperature-regulated and differentially expressed genes that represent new targets for further studies. In addition, we have established cell models that will allow the comparison of different adipose tissue depots at an in vitro level. From these investigations, we have demonstrated that neuregulin 4 (Nrg4) is a novel cold-induced adipokine that promotes neurite outgrowth.

MATERIALS AND METHODS

Mice

Cold exposure experiments. Groups of four 10-wk-old female 129Sv mice (Charles River, Milan, Italy) were housed separately and kept at 22°C. Subsequently, one group of mice was placed at 28°C and another one at 6°C for 10 days. Another group was kept at 22°C. Animals were singly caged with a 12:12-h light-dark cycle and free access to standard chow diet and water. At the end of the treatment, the body weights of single animals did not change significantly either during cold exposure or in warm acclimatization. Mice were culled as reported previously (7), and adipose tissues (interscapular BAT and subcutaneous, mesenteric, and gonadal WAT) were collected. Care and handling were in accordance with institutional guidelines. Procedures were approved by the Ethics Committee for Animal Experiments at the University of Ancona (11 Gen. 2007, protocol no. 585).

β-Adrenoceptor agonist administration experiments. Two groups of four 10-wk-old C57BL/6 female mice kept at 22°C were given an intraperitoneal injection either with saline (200 μl) or CL316,243 (1 mg/kg body wt in 200 μl of saline) 5 h after injection mice were culled by cervical dislocation and adipose tissues collected.

Tissue panel. FVB/N female mice kept under normal housing conditions were culled by cervical dislocation and the different tissues collected. Experiments were carried out in accordance with UK Home Office regulations.

Immunohistochemistry and Immunocytochemistry

Immediately after removal, adipose tissues were fixed overnight by immersion at 4°C in 4% paraformaldehyde. Paraﬁnin-embedded dewaxed sections were stained with hematoxylin and eosin or incubated with anti-UCP1 (cat. no. 10983; Abcam) or anti-Nrg4 (anti-127; see Ref. 24) according to the avidin-biotin complex method (27). Staining was never observed when the primary antibody was omitted.

RNA Extraction and qRT-PCR

Total RNA was extracted with Trizol. RNA for microarray analysis was puriﬁed using Qiagen RNeasy mini columns. The expression of target genes was determined using SYBR green reagent and gene-speciﬁc primers (sequences available on request). Relative expression levels were normalized to β-actin.

Microarray Analysis

Gene expression microarray was performed by Almac diagnostics UK using GeneChip Mouse Genome 430A 2.0 Array (Affymetrix) on RNA extracted from interscaped BAT and subcutaneous and mesenteric WAT from mice exposed to 28 or 6°C. Three biological replicates were used. The analysis was conducted using R programming language and Bioconductor (55). After background adjustment, summarization, and normalization of the raw intensities, the data were analyzed using affy (19) and limma (14). Pairwise comparisons between conditions were performed. A linear model was fitted to the expression values of each probe, modiﬁed to account for the replicated samples and assuming no interaction between the genes. The moderated t-statistic was computed for each probe and for each contrast providing P values that were corrected for multiple testing using the Benjamini and Hochberg’s method to control the false discovery rate (10). Probes with adjusted P values of <0.01 were considered statistically significant. The microarray data have been deposited in NCBI’s Gene Expression Omnibus (15) and are accessible through GEO Series accession no. GSE51080 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51080). Heatmaps were produced based on the Euclidian distance. Pathway analysis was generated by Ingenuity IPA software.

Protein Expression

Tissues and cultured cells were lysed in Laemmli loading buffer, and proteins were electrophoresed on a 10% SDS-PAGE gel and transferred to PVDF membrane. Membranes were probed with specific antibodies UCP1 (cat. no. U6382; Sigma), cell death-inducing DNA fragmentation factor α-subunit-like effector A (CIDEA; cat. no. AB16922; Chemicon), p-Akt (Ser473), and t-Akt, all from cell-signaling technologies and β-actin (Sigma).

Preparation of adipocytes vs. stromal vascular fraction-enriched fractions

Adipose tissues from four mice were cut into 2-mm pieces and digested in serum-free DMEM-F-12 culture medium containing 1 mg/ml collagenase A (Roche) and 10 mg/ml DNAse (Roche). After digestion, the cells were separated by centrifugation at 170 g for 10 min, and mature adipocytes (floating fraction) and stromal vascular fraction (SVF; in the cell pellet) were collected separately for RNA extraction.

Cell Culture

Generation of immortalized cell lines. Primary cultures of brown adipose and subcutaneous, mesenteric, and gonadal white adipose tissues were generated by first digesting the tissue and pelleting the SVF, as described above. Preadipocytes were puriﬁed by collecting the cells that passed through 100 μm of mesh. Next, cells were passed through strainers of 70- or 25-μm mesh size for brown and white
preadipocytes, respectively, and were plated and cultured in DMEM-F-12, 10% FBS, and 1% antibiotic-antimycotic. After culture for 2 days, preadipocytes were immortalized by retroviral-mediated expression of temperature-sensitive SV40 large T-antigen H-2kb-tsA58 (a gift from P. Jat). Cells were cultured at 33°C and selected with G418 (100 mg/ml) for 2 wk and then maintained in 50 µg/ml G418. Experiments were performed between passages 12 and 22. Cells were differentiated as described previously (22, 39).

PC12-HER4. The PC12-HER4 cell line (24) was cultured in RPMI-1640 supplemented with 10% horse serum and 5% fetal bovine serum. Cells were plated on poly-1-lysine-coated glass coverslips and exposed to unconditioned DMEM-F-12, 10% FBS medium, or conditioned medium from differentiated adipocytes.

Metabolic Studies

9,10-[3H]palmitate oxidation. Assessment was performed as reported previously (18). Briefly, differentiated adipocytes were starved in glucose-deprived DMEM for 1 h. Cells were then incubated at 37°C in DMEM containing 0.3 mM palmitate (9,10-[3H]palmitate, 12 µCi/ml), 2% BSA, 0.25 mM t-carnitine, and 3 mM glucose for 3 h. Palmitate oxidation was assessed by measuring 3H2O produced in the incubation medium. Data were normalized to DNA content/well measured with a Fluorescent DNA Quantitation Kit (Bio-Rad).

Glucose uptake. Glucose uptake was measured using 2-[5-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG; Molecular Probes). Differentiated adipocytes in 96-well plates were serum starved for 3 h. Then cells were incubated in HBSS buffer with or without insulin (100 nM) for 30 min. Glucose uptake was initiated by the addition of HBSS containing 500 µM 2-NBDG and 2.5 mM deoxyglucose. After 15 min, plates were washed with cold HBSS buffer containing 25 mM glucose. Cells were lysed, and fluorescence was read at 466 nm. Normalization was performed to protein content.

Nrg4 ELISA

NRG4 levels in culture medium were determined by ELISA (SEC147Mu; Caltag MedSystems), following the manufacturer’s protocol.

IL-6 ELISA

Antibodies and standards were sourced from R & D Systems, and the ELISA was performed according to the manufacturer’s instructions. Data were normalized to DNA content/well measured with a Fluorescent DNA Quantitation Kit (Bio-Rad).

Nrg4 Knockdown

Conditionally immortalized BAT cells were transfected with shRNA (short-hairpin RNA) targeting Nrg4 (pSuperior-shNrg4). Oligonucleotides (shNrg4 forward, 5'-GATCCCCUGGGGGGAAUUUGUUAUGUGTCTAAGGACACUAACAAACCCCAATTCTTTA-3'; and shNrg4 reverse, 5'-AGCTTAAAAGGGAAGUUUGGAAUUGUGUGUGCTCTTGAAACACUAACAAACCCCAAGGG-3') were annealed and ligated into pSuperior vector. Cells stably expressing the shRNA were achieved by selection with puromycin.

Statistical Analysis

ANOVA tests were performed, and only P values <0.05 considered statistically significant. In all figures, the data are the average of a minimum of three independent replicates ± SE.

Motif Detection in Upstream Flanking Regions of Differentially Expressed Genes

Lists of differentially expressed genes from the expression experiments were ordered using fold change, and Ensembl Gene IDs were obtained for lists of interest. The Ensembl Gene IDs were subsequently submitted to BioMart (Ensembl Genes 72, Mus musculus GRCm38.p1 dataset) to retrieve the corresponding 1,000-bp upstream flanks of the transcripts of the genes of interest from the genome annotation. To remove sequence repeats arising from overlapping transcript variants, the FASTA sequences were assembled into contiguous sequences with CAP3. Mixed contiguous sequences composed of flanks from more than one gene were verified to identify overlaps generated in CAP3 due to sequence similarity alone rather than contiguity. Such false mixed contiguous sequences were removed from the set of contiguous sequences. The original flanking regions making up the false mixed contiguous sequences were then collected, and a contiguous sequence was rebuilt if there was more than one transcript-flanking region for a particular gene, whereas singlets were simply added to the final set. The final set of sequences was then collated, which included the initial true contiguous sequences, the singlets, and the singlets or contiguous sequences which arose from the false mixed contiguous sequences. The resulting FASTA sequence file was submitted as a single cluster to MEME-LaB to detect 10 motifs between six and 12 base pairs, with the promoter maximum length adjusted to accommodate the longer contiguous sequences.

RESULTS

Fat Depots Vary in Their Response to Cold

To study the ability of different adipose tissue depots to respond to changes in temperature, 129Sv female mice were exposed to temperatures of 28 or 6°C. This mouse strain is reported to be diabetes and obesity resistant due to a greater amount of active BAT (3). It has a greater amount of adipocytes expressing UCP1 in different adipose depots and has been used for several studies addressing cold-induced transdifferentiation of adipose tissues (7, 40). Global changes in expression were monitored by microarray analysis of BAT and inguinal subcutaneous and mesenteric WAT. BAT showed the most dramatic changes upon prolonged cold exposure, with 11,180 gene probes sets differentially expressed (P < 0.01) (Fig. 1A). In subcutaneous WAT, 2,178 probes were altered, but only eight were altered in mesenteric WAT.

By considering the up- and downregulated probes separately, we identified the most significantly differentially expressed pathways in each tissue. The top 20 pathways for BAT and subcutaneous WAT are shown in Fig. 1B. The number of genes altered in the mesenteric depot was too low to be used for pathway analysis. In BAT, the top upregulated pathways involve protein degradation (protein ubiquitination), metabolism (fructose, mannose, fatty acid, glucose, and propanoate metabolism), reduced power generation (pentose phosphate pathway), signal transduction (thyroid hormone receptor/retinoid X receptor regulation, Toll-like receptor (TLR) activation, AMPK, IL-17, PI3K/Akt), and oxidative stress regulation. Among the downregulated probes, the most significantly altered pathways are cell cycle- and growth regulation-related pathways (purine metabolism, DNA methylation, cell cycle G1/S checkpoint, DNA damage repair) as well as signaling pathways (mTOR, estrogen, RAR, androgen, and VEGF signaling). On analysis of the subcutaneous data set, it becomes evident that this particular depot shows a shift toward a brown or brite phenotype when animals are exposed to 6°C. Most of the top upregulated pathways are those that are highly expressed in BAT (oxidative phosphorylation, citrate cycle, fatty acid metabolism, ubiquinone synthesis, pyruvate metabolism).

Downregulated probes belong mostly to pathways related to
Fig. 1. Global expression analysis of brown adipose tissue (BAT) and subcutaneous and mesenteric white adipose tissue (WAT) in mice exposed to cold. A: global mRNA expression was measured in BAT and subcutaneous and mesenteric WAT depots of mice exposed at either 28 or 6°C using Affymetrix 2.0 ST microarrays. A row-centred heat map of hierarchical clustering carried out on the differentially expressed gene probes at a 5% false discovery rate is shown. Probe sets are colored according to the average expression level across all samples, with green denoting a lower expression level and red denoting a higher expression level.

B: top pathways that were altered by cold exposure in BAT and subcutaneous WAT. The bars represent significance, and the ratio of genes altered out of the total genes per pathway is indicated. Pathway analysis was performed using up- and downregulated probes separately. mTOR, mammalian target of rapamycin; EIF, eukaryotic initiation factor; NRF2, nuclear factor-E2-related factor; PI3K, phosphatidylinositol 3-kinase; CNTF, ciliary neurotrophic factor; RAR, retinoic acid receptor.
Fig. 2. Global expression comparison of BAT and subcutaneous (sWAT) and mesenteric WAT (mWAT) at 28°C. A: global mRNA expression was measured in BAT, sWAT, and mWAT depots of mice housed at 28°C, using Affymetrix 2.0 ST microarrays. Shown is a row-centred heat map of hierarchical clustering carried out on the differentially expressed gene probes at a 10% false discovery rate. Probe sets are colored according to the average expression level across all samples, with green denoting a lower expression level and red denoting a higher expression level. B: top pathways that are enriched in each of the different depots compared with each other. The bars represent significance, whereas ratio shows the genes altered out of the total genes per pathway. Pathway analysis was performed using up- and downregulated probes separately.
the immune system (lipid antigen presentation, natural killer cell signaling, antigen presentation), signaling, and endoplasmic reticulum stress.

**Intrinsic Differences of WAT and BAT Depots at Thermoneutrality**

Using the microarray data, we analyzed the intrinsic differences between each adipose depot at 28°C. In Fig. 2A, the heat maps for each of the comparisons are shown. Comparison of BAT to subcutaneous and mesenteric WAT indicated that 25,625 and 30,242 probes, respectively, were differentially expressed, whereas only 9,647 probes were differentially expressed for subcutaneous vs. mesenteric. As expected, these data show that WAT depots are more similar to each other than to BAT.

The most highly expressed pathways in BAT were those involved in energy expenditure, metabolism, and signaling (Fig. 2B), with those enriched in WAT related to the immune system, including T cell activation, antigen presentation, complement system activation, and lymphocyte apoptosis. When comparing subcutaneous to mesenteric WAT, the enriched pathways in subcutaneous WAT are related to neuronal sig-

**Table 1. Top gene probes up- and downregulated after cold exposure in BAT and subcutaneous and mesenteric WAT**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>FC</th>
<th>Adjusted P Value</th>
<th>Gene Name</th>
</tr>
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<tr>
<td>Elov13</td>
<td>76.76</td>
<td>&lt;0.001</td>
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<td>Bmp8b</td>
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<td>&lt;0.001</td>
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<td>4933433H22Rik</td>
<td>15.12</td>
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<td>Serpina12</td>
<td>14.88</td>
<td>&lt;0.001</td>
<td>Serine peptidase inhibitor, clade A member 12</td>
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<tr>
<td>Gyk</td>
<td>11.83</td>
<td>&lt;0.001</td>
<td>Glycerol kinase</td>
</tr>
<tr>
<td>Ncan</td>
<td>11.71</td>
<td>&lt;0.001</td>
<td>Neurocan</td>
</tr>
<tr>
<td>Rim2</td>
<td>10.38</td>
<td>&lt;0.001</td>
<td>Regulating synaptic membrane exocytosis 2</td>
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<td>Scl25a34</td>
<td>9.62</td>
<td>&lt;0.001</td>
<td>Solute carrier family 25, member 34</td>
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<tr>
<td>Sgk2</td>
<td>9.51</td>
<td>&lt;0.001</td>
<td>Serum/glucocorticoid regulated kinase 2</td>
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**Table 1.** Subcutaneous WAT 6°C vs. 28°C Upregulated

<table>
<thead>
<tr>
<th>Gene Symbol</th>
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<td>Fabp3</td>
<td>10.21</td>
<td>&lt;0.001</td>
<td>Fatty acid-binding protein 3</td>
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<td>Cpt1b</td>
<td>9.88</td>
<td>&lt;0.001</td>
<td>Carnitine palmitoyltransferase 1b, muscle</td>
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<td>Cox7a1</td>
<td>9.21</td>
<td>&lt;0.001</td>
<td>Cytochrome c oxidase, subunit VIIa 1</td>
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<td>Kng1</td>
<td>7.84</td>
<td>&lt;0.001</td>
<td>Kininogen 1</td>
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<td>Otop1</td>
<td>7.49</td>
<td>&lt;0.001</td>
<td>Otopetrin 1</td>
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<tr>
<td>Ppara</td>
<td>7.48</td>
<td>&lt;0.001</td>
<td>Peroxisome proliferator-activated receptor-α</td>
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</table>

**Table 1.** Subcutaneous WAT 6°C vs. 28°C Downregulated

<table>
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<td>Nnat</td>
<td>16.72</td>
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<td>neauramin</td>
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<td>Kera</td>
<td>5.15</td>
<td>&lt;0.001</td>
<td>Keratocan</td>
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<td>Defb1</td>
<td>5.06</td>
<td>0.002</td>
<td>Defensin-β1</td>
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<td>Gm266</td>
<td>4.98</td>
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<td>predicted gene 266</td>
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<td>Co9a1</td>
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<td>Collagen type IX, α1</td>
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<td>Htr4</td>
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<td>Scl15a2</td>
<td>3.24</td>
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<td>Bdnf</td>
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<td>Adamts2</td>
<td>3.10</td>
<td>1.70E-04</td>
<td>A disintegrin-like metallopeptidase throm-bospondin type1, motif2</td>
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</table>

**Table 1.** Mesenteric WAT 6°C vs. 28°C Upregulated

<table>
<thead>
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<th>Gene Symbol</th>
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<tr>
<td>Ucp1</td>
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<td>Cabc1</td>
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<td>&lt;0.001</td>
<td>Chaperone, ABC1 activity of bc1 complex like</td>
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<td>Acsf2</td>
<td>1.77</td>
<td>&lt;0.001</td>
<td>Acyl-CoA synthetase family member 2</td>
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**Table 1.** Mesenteric WAT 6°C vs. 28°C Downregulated

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<td>LOC100046232</td>
<td>2.66</td>
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<td>Nuclear factor interleukin 3, regulated</td>
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<td>Tmem182</td>
<td>1.84</td>
<td>&lt;0.001</td>
<td>Transmembrane protein 182</td>
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BAT, brown adipose tissue; WAT, white adipose tissue; FC, fold change.
naling, whereas the most prominent pathways in mesenteric WAT seem to be related to interleukin production and signal- ing as well as carbohydrate and amino acid metabolism.

Identification of Differentially Expressed Genes Sensitive to Temperature

From the microarray analysis, we aimed to identify genes that were significantly altered in expression by temperature comparisons of the depots at 28°C are presented in Table 2. We chose a set of genes for further study that were more highly expressed in BAT compared with WAT depots and were also increased in subcutaneous WAT following cold exposure. This

<table>
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<th>Gene symbol</th>
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<td>Myo5c</td>
<td>65.24</td>
<td>&lt;0.001</td>
<td>Myosin VC</td>
</tr>
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<td>Myh4</td>
<td>64.22</td>
<td>&lt;0.001</td>
<td>Myosin, heavy polypeptide 4, skeletal muscle</td>
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Subcutaneous 28°C vs. Mesenteric 28°C Upregulated

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Subcutaneous 28°C vs. Mesenteric 28°C Downregulated

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Table 2. Top gene probes up- and downregulated when comparing BAT, subcutaneous WAT, and mesenteric WAT at thermoneutrality
group included genes involved in several different processes. Nrg4 is a regulator of neuronal development (23), and Bmp8b regulates thermogenesis in BAT (62). The G protein-coupled receptor (GPCR) Gpr120 is linked with obesity (29), and Rgs7 (4) has the potential to affect the signaling through many GPCRs important for BAT function. Slc27a2 (26) and Letm1 (60) have key roles in lipid metabolism and mitochondrial function, respectively. We also identified the uncharacterized transcript 9030619P08RIK for further study, as it is highly regulated and could be a potential cell surface marker of brown adipocytes. This gene shares a high homology with the Ly-6 protein family and is found in the vicinity of a genomic region that contains the genes for this family (6). Hence, we term it cold-induced lymphocyte antigen 6-like (Cil6l). Neuronatin (Nnat), a gene that has been involved in calcium signaling and ER stress and adipose biology (20, 32), was selected because it was downregulated in cold conditions in BAT and subcutaneous WAT.

Prior to the microarray, we determined the expression of UCP1, a classic cold-induced target gene in BAT by qRT-PCR in the different adipose tissues. We included another visceral fat depot, the gonadal WAT, and tissues from animals housed at 22°C (Fig. 3 A). UCP1 was induced in all depots after exposure to 6°C compared with either 28 or 22°C, with the subcutaneous depot showing the largest increase. We also measured the expression of Cidea, which was increased in subcutaneous WAT but only modestly in BAT. Hematoxylin and eosin staining showed a large increase in multilocular cells of the subcutaneous depot upon cold exposure (Fig. 3B).

Protein expression of UCP1 was monitored in the different depots at 5 h after injection of CL-316,243, a β3-receptor agonist (Fig. 4). UCP1 was increased in all depots compared with the saline-injected groups. We also measured the expression of several other genes involved in thermogenesis and lipid metabolism by qRT-PCR in the different adipose tissues (Fig. 4). The expression of these genes was induced in all depots after exposure to cold, with the subcutaneous depot showing the largest increase. We also measured the expression of several other genes involved in thermogenesis and lipid metabolism by qRT-PCR in the different adipose tissues (Fig. 4). The expression of these genes was induced in all depots after exposure to cold, with the subcutaneous depot showing the largest increase.

Fig. 3. Expression of selected genes induced by cold exposure or by a β3-adrenergic-specific agonist in different adipose depots. A: mRNA expression of uncoupling protein 1 (UCP1) and cell death-inducing DNA fragmentation factor α-subunit-like effector A (Cidea) in BAT, sWAT, gonadal WAT (gWAT), and mWAT of mice exposed to 28, 22, or 6°C. B: hematoxylin and eosin staining (H & E) and UCP1 immunohistochemistry. UCP1-immunoreactive brown adipocytes interspersed among unilocular white adipocytes of subcutaneous adipose tissue of mice exposed to 28 or 6°C. C: mRNA expression of genes identified by microarray in BAT, sWAT, gWAT, and mWAT of mice exposed to 28, 22, or 6°C. D: mRNA expression of selected genes in different adipose depots of mice injected intraperitoneally with the selective β3-receptor agonist CL-316,243 (1 mg/kg body wt) after 5 h; n = 4. All mRNA data are expressed as fold induction compared with BAT of mice either at 28°C or injected with saline. Bars represent the means ± SE of at least 3 mice/group, and significant differences are shown. *P < 0.05; **P < 0.005; ***P < 0.001.

Fig. 4. Adipocyte-selective expression of temperature-regulated genes. mRNA expression of in cells from the stromal vascular fraction (SVF) or mature adipocytes prepared from interscapular BAT, sWAT, gWAT (Gon), and mWAT. Perilipin protein levels from gWAT are shown as an indicator of the quality of the fractionation (top right). Nrg, neuregulin; Nnat, neuronatin; Cil6l, cold-induced lymphocyte antigen 6-like; Letm1, leucine zipper EF hand-containing transmembrane protein 1.
depots by immunohistochemistry. Figure 3B shows staining of subcutaneous WAT, UCP1 staining increased with exposure to low temperatures in all adipose depots studied (data not shown).

We next validated expression of our genes of interest (Fig. 3C). At both 28 and 22°C, Slc27a2 and Letm1 expression was higher in BAT compared with the other depots, and Bmp8b expression was higher in BAT and subcutaneous WAT, whereas Nrg4, GPR120, and Rgs7 were highly expressed in BAT and gonadal WAT depots. In the gonadal depot, the relatively high levels of these genes are likely due to constitutively present brown adipocyte pockets observed in the 129Sv strain even at 28°C (57). Cil6l was expressed largely in subcutaneous WAT, whereas Nnat was preferentially expressed in gonadal WAT, followed by subcutaneous and mesenteric WAT and BAT. Upon cold exposure, we found genes with expression induced in all depots (Rgs7, Letm1), genes with expression induced in BAT and subcutaneous and gonadal WAT (Nrg4, Gpr120), genes induced only in BAT and subcutaneous WAT (Bmp8b, Cil6l, Slc27a2), and a gene that was reduced in all depots (Nnat).

To investigate whether the cold-regulated genes were affected directly by the \( \beta_3 \)-adrenergic signaling pathway, mice were treated with the \( \beta_3 \)-specific adrenergic agonist CL-316,243. Examination of the expression of the cold-responsive genes (Fig. 3D, right) revealed that only Bmp8b (very modestly) and GPR120 mRNAs were significantly induced by acute adrenergic treatment and only in BAT. These results indicate that many of the genes that are upregulated in WAT result from long-term remodeling effects rather than rapid direct transcriptional events and are likely to be important for brite cell function rather than driving the “browning” process. Alternatively, the genes may initially be refractory to the \( \beta_3 \)-stimulus.

Because adipose tissues are composed of several cell types, we determined whether the identified genes were expressed in mature adipocytes or nonadipocyte cells that constitute the SVF. Fractionation of the different depots showed that all the genes were preferentially expressed in either brown or white mature adipocytes (Fig. 4).

### Gene Expression in Immortalized Cell Lines Generated From Different Adipose Tissue Depots

Considering that all of the genes we identified were preferentially expressed in mature adipocytes, we next investigated whether their expression was maintained in cells in vitro. We took the approach of immortalizing adipocyte precursors from each adipose depot. This allows the comparison of adipocytes from depots where both large and small numbers of precursors can be obtained, using fewer mice. First, we examined whether the in vitro-differentiated adipocytes retained the features that define each depot. For all the cell lines, 80–100% of cells were differentiated as judged by Oil Red O stain (Fig. 5A). The ability to differentiate into adipocytes was maintained after more than 30 passages. At the expression level, the preadipocyte marker Pref-1 and mature adipocyte markers aP2 and PPAR\( \gamma \) were downregulated or highly induced, respectively, upon differentiation in the four cell lines (Fig. 5B). The brown adipocyte marker UCP1 (Fig. 5A) was highly induced in BAT cells after differentiation and to a much lower extent in white adipocyte cells. Expression of UCP1 was almost undetectable in gonadal and mesenteric WAT cells. We then analyzed expression of the identified set of temperature or differentially expressed genes throughout differentiation (Fig. 5C). Nnat and Slc27a2 mRNA levels were undetectable at different time points in all cell lines. Expression of the other identified genes was induced upon differentiation to a higher or lower extent in all cell lines. Nrg4, Rgs7, and Letm1 were more highly expressed in differentiated BAT cells compared with the white adipocyte lines. Cil6l was highly expressed in differentiated subcutaneous cells and to a lower extent in gonadal. We next investigated gene regulation due to \( \beta_3 \)-adrenergic signaling. We exposed differentiated adipocytes to an acute (5 h) treatment with CL-316,243. This compound was able to induce UCP1 and PGC-1\( \alpha \) mRNA (Fig. 5D), showing that the \( \beta_3 \)-adrenergic signaling pathway is fully functional. Regarding the genes identified from the array, we obtained results similar to the in vivo CL-316,243 treatment; GPR120 was the most sensitive to acute adrenergic activation in BAT and subcutaneous cells. In general, Bmp8b was present at low levels in adipocyte cell lines, unlike adipose tissues. However, it was present at higher levels in differentiated brown compared with white adipocytes and was induced by CL-316,243 only in the brown adipocytes (Fig. 5D). Nrg4 and Letm1 showed modest induction of mRNA expression in BAT cells and a tendency to increase in subcutaneous cells but not in any of the others. These results highlight that pathways in addition to the \( \beta_3 \)-adrenergic signaling pathway are necessary for the induction of certain genes (Cidea, Nrg4, Rgs7) upon cold exposure, whereas others (UCP1, PGC-1\( \alpha \), GPR120) are regulated directly by this pathway.

We also performed a series of functional metabolic assays to investigate whether the differences observed at the transcriptional level had physiological relevance in our cell lines. Basal \( \beta \)-oxidation was highest in BAT and subcutaneous WAT (Fig. 6A). Because cold activates BAT \( \beta \)-oxidation (65), we treated the cells with 10 \( \mu \)M CL-316,243 for 5 h. We detected an increase in the \( \beta \)-oxidation by BAT and subcutaneous WAT cells, whereas it remained unchanged in gonadal and mesenteric WAT cells. Basal glucose uptake, assessed with a fluorescent glucose analog (2-NBDG) in mature adipocytes, was the highest in gonadal WAT cells, followed by mesenteric and subcutaneous WAT and BAT (Fig. 6B). Considering the recent findings by PET scans, surprisingly, BAT had the lowest basal glucose uptake. This cannot be explained by the expression levels of the glucose transporters GLUT1 and GLUT4 that were present.
at higher levels in BAT compared with WAT cell lines (Fig. 6B) and is likely due to differences between white and brown adipocytes in the pathways controlling glucose uptake. Upon insulin stimulation, glucose uptake increased in gonadal and subcutaneous WAT cells by 30 and 20%, respectively, whereas CL-316,243 treatment increased BAT adipocyte glucose uptake (Fig. 6B), showing that β-adrenergic stimulus is more important than insulin for BAT glucose uptake. Furthermore, Akt phosphorylation was increased by insulin stimulation in all cell lines very potently in WAT and subcutaneous WAT, indicating that insulin signaling was intact in the differentiated adipocytes (Fig. 6B).

Adipocytes are an important source of inflammatory cytokines (34), and our microarray analysis revealed that WAT depots preferentially expressed gene pathways associated with inflammatory processes. Because previous investigations showed that differentiated adipocytes from human primary cultures were highly sensitive to LPS-stimulated secretion of IL-6 (11), we investigated the inflammatory response of differentiated adipocytes derived from distinct adipose tissues. Basal IL-6 secretion was detected in all cell lines except for BAT, and stimulation with LPS increased IL-6 production in a dose-dependent manner in subcutaneous and gonadal WAT cells (Fig. 6C). The lack of response of the mesenteric adipocytes was somewhat surprising given the link between visceral depots and inflammatory activity. Similar levels of TLR4 mRNA, the receptor for LPS, were detected in all cell lines following differentiation and induced ≥2,000-fold in all of the cell lines in differentiated compared with preadipocytes (data not shown).

This is the first time immortalized cell lines from four different adipose depots (interscapular BAT and subcutaneous, gonadal, and mesenteric WAT) have been generated and compared. Results demonstrate that it is possible to maintain depot-specific differences in expression and physiology in vitro systems.

**Genes and Motifs Defining Browning of White Fat**

To characterize the transcription profile of WAT subject to browning in response to cold, we first prepared a list of genes that were enriched in both BAT vs. subcutaneous WAT and BAT vs. mesenteric WAT (comparisons at 28°C), which provides BAT genes. Next, we determined which genes from this list were also increased in subcutaneous WAT by cold exposure. The genes of this brite transcriptome are presented in Table 3. This stringent analysis confirmed the association of UCP1, PGC-1α, Cidea, Plin5, PPARγ, and Otop1 with...
Table 3. List of genes that define the “browning” of cold-activated white adipose tissue

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**Transmitters**

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**Genes listed were upregulated in subcutaneous WAT and more highly expressed in both BAT versus subcutaneous WAT and BAT versus mesenteric WAT.**

browning of adipose tissue and also revealed additional genes that were part of the brown/brite transcription “fingerprint,” including GPR120, Nrg4, Rgs7, and Letm1. To investigate the regulatory motifs associated with brite genes, we took the 1,000-bp upstream flanking region from the top 308 sequences. After the removal of duplicates and rebuilding of mixed contigs, a total of 471 sequences were submitted to MEME-LaB. The 10 most significant motifs between six and 12 bp were identified and their similarity to known eukaryotic transcription factor-binding sites determined (Fig. 7). For motif 2, which was present in 100% of promoter sequences, the transcription factors Klf4, CAC-binding protein, Pu.1, and Sp1 were the nearest fit from their position-specific scoring matrix (PSSM). Other transcription factors that were identified as fitting the motifs included C/EBPβ, p53, Pax6, Pax2, BCL-6, ATF-5, Kid3 (Zfp354c), and the nuclear receptors SF1, LRH-1, and HNF-4. Examination of the microarray data reveals that C/EBPβ, BCL-6, SF1, Pax6, and ATF-5 are more highly expressed in brown vs. white adipose tissues at thermoneutrality. This highlights the potential of these factors through binding to their respective motifs to affect brown adipocyte gene expression.

Finally, the microarray data was analyzed to determine which transcripts were temperature regulated but not specifically associated with higher levels in BAT. We generated a list of genes that were more highly expressed at 6 vs. 28°C in subcutaneous WAT but not more highly expressed in BAT vs. mesenteric WAT or BAT vs. subcutaneous WAT (Table 4). Remarkably, of this list of 53 cold-induced genes in subcutaneous WAT, 40 were also cold-induced in BAT. Genes that were increased in both WAT and BAT in response to cold include Cil6l, Aca1b1, Dbp, Drd1a, Gys2, Lipf, Pla2g2e, and Sphk2. They are likely to be important for changes required only in response to cold and may be required for elevation of metabolic activity that will occur in both WAT and BAT in cold conditions. Although these genes are not more highly expressed in BAT vs. WAT at thermoneutrality, they may still be important for the brite program.

**Nrg4 is a Brown Adipocyte Adipokine That Promotes Neurite Outgrowth**

Nrg4 was one of the genes for the group that define the brite transcription signature and was selected for further study. It showed an expression pattern similar to the BAT marker gene Cidea in that expression was greater in BAT vs. WAT and that cold stimulated a large increase in WAT, whereas only a small increase was detected in BAT. Along with Nrg1, -2, and -3, it is a member of the epidermal growth factor family of proteins that are involved in normal development and in the pathology of many diseases. Analysis of a panel of mouse tissues revealed that Nrg4 was highly expressed in adipose tissues, being the highest in BAT, followed by gonadal and subcutaneous WAT (Fig. 8B). For in vitro studies, we determined expression of Nrg4 mRNA in our immortalized BAT cell line. Expression progressively increased upon differentiation treatment, being the highest after 8 days (Fig. 8C), confirming adipocyte-specific expression (Fig. 5C). Efficient differentiation was confirmed by the induction of the adipocyte marker aP2 (Fig. 8C). To test whether NRG4 was secreted into the culture media and whether this had any effect in promoting neurite outgrowth, conditioned medium of mature brown adipocytes was used to treat PC12 cells stably expressing ErbB4 (25). NRG4 was detected by ELISA in conditioned medium.
### Motif Features

<table>
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<td>2.8e-61</td>
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### PSSM Distance Score Factor

| HK-00008 | 8.1 | KLF4 |
| EVE-0008 | 8.1 | TFIH |
| S-000067 | 8.1 | CAC-binding protein |
| S-000477 | 9.4 | Sp1 |
| MA-00391 | 8.4 | KLF4 |
| M00706 | 9.0 | TFIH |
| M00720 | 10.0 | CAC-binding protein |
| M00658 | 10.6 | Pu.1 |
| M00008 | 10.9 | Sp1 |
| MA-03591 | 12.9 | |
| M00633 | 13.5 | |
| S-000143 | 13.7 | |
| M00213 | 13.9 | |
| RO2164 | 14.4 | HIF1 |
| M00979 | 6.2 | Pax6 |
| R14701 | 9.3 | SF1 |
| M01142 | 11.2 | URH-1 |
| M01171 | 11.3 | BCL-6 |
| M00808 | 11.6 | PAX |
| M00933 | 2.1 | Sp1 |
| M01273 | 2.2 | Sp4 |
| MA-00792 | 2.2 | Sp1 |
| M00932 | 2.4 | Sp1 |
| M00931 | 2.6 | Sp1 |
| MA-02671 | 7.7 | |
| MA-04021 | 8.3 | |
| M00720 | 9.4 | CAC-binding protein |
| M01030 | 9.7 | |
| RO2170 | 9.8 | CEBPbeta |
| S-000472 | 12.5 | |
| MA-02601 | 13.6 | |
| S-000308 | 14.1 | |
| S-000245 | 14.9 | |
| M00486 | 15.1 | Pax2 |
| S-000474 | 13.4 | |
| M00761 | 14.5 | p53 |
| M01033 | 15.1 | HNF-4 |
| M00629 | 15.2 | |
| M01032 | 15.3 | HNF-4 |
| S-000482 | 11.7 | |
| MA-03731 | 12.0 | |
| MA-03541 | 12.3 | |
| M01160 | 13.0 | Kid3 |
| MA-03331 | 13.4 | |
from differentiated brown adipocytes and was undetectable in unconditioned medium and medium from preadipocytes (Fig. 8D). Conditioned medium induced neurite outgrowth (measured by staining with anti-Tuj1 antibody) after 24 h in a dose-dependent manner (Fig. 8E). This effect was specific for Nrg4, as neurite outgrowth was prevented in conditioned medium from adipocytes in which it was knocked down by 75% by shRNA interference (Fig. 8F). Thus, we can

Table 4. Genes that are induced by cold in subcutaneous WAT and are not more highly expressed in BAT versus subcutaneous WAT or BAT versus mesenteric WAT

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Induced by Cold in BAT</th>
<th>Gene Function</th>
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<tr>
<td>4921536K21Rik</td>
<td>RIKEN cDNA 4921536K21 gene</td>
<td>Yes</td>
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<tr>
<td>9030619P08Rik</td>
<td>RIKEN cDNA 9030619P08 gene</td>
<td>Yes</td>
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<tr>
<td>Abca6</td>
<td>ATP-binding cassette, sub-family A (ABCA1), member 6</td>
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<tr>
<td>Abhd1</td>
<td>Abhydrolase domain containing 1</td>
<td>Yes</td>
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<td>Acatal1b</td>
<td>Acetyl-Coenzyme A ayltransferase 1B</td>
<td>Yes</td>
</tr>
<tr>
<td>Acox1</td>
<td>Acyl-coenzyme A oxidase-like</td>
<td>Yes</td>
</tr>
<tr>
<td>Adrn1</td>
<td>Adhesion regulating molecule 1</td>
<td>Yes</td>
</tr>
<tr>
<td>Apoc2</td>
<td>Apolipoprotein C-II</td>
<td>Yes</td>
</tr>
<tr>
<td>Apoc4</td>
<td>Apolipoprotein C-IV</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Apold1</td>
<td>Apolipoprotein L domain-containing 1</td>
<td>Yes</td>
</tr>
<tr>
<td>Aqp11</td>
<td>Aquaporin 11</td>
<td>Yes</td>
</tr>
<tr>
<td>Atr</td>
<td>Attractin</td>
<td>Yes</td>
</tr>
<tr>
<td>C2cd2l</td>
<td>C2 calcium-dependent domain containing 2-like</td>
<td>Yes</td>
</tr>
<tr>
<td>Catsper3</td>
<td>Cation channel, sperm associated 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Ccny</td>
<td>Cyclin Y</td>
<td>Yes</td>
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<tr>
<td>Chrarna2</td>
<td>Cholinergic receptor, nicotinic, alpha polypeptide 2</td>
<td>Yes</td>
</tr>
<tr>
<td>Cir1</td>
<td>Corepressor interacting with RBPI, 1</td>
<td>Yes</td>
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<tr>
<td>Clnd22</td>
<td>Claudin 22</td>
<td>Plasma membrane</td>
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<tr>
<td>Cnml</td>
<td>Carboxymethylenbutenolidase-like (Pseudomonas)</td>
<td>Yes</td>
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<tr>
<td>Crtac1</td>
<td>Cartilage acidic protein 1</td>
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<tr>
<td>Cyp4a10</td>
<td>Cytochrome P450, family 4, subfamily a, polypeptide 10</td>
<td>Yes</td>
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<tr>
<td>Dpp</td>
<td>D site albumin promoter binding protein</td>
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<td>Dcst1</td>
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<tr>
<td>Drd1a</td>
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<tr>
<td>Fbpi2</td>
<td>Fatty acid-binding protein 12</td>
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<td>Fndc8</td>
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<td>Glyc2</td>
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<td>Katna1</td>
<td>Katanin p60 (ATPase-containing) subunit A</td>
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<td>Kdh2</td>
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<tr>
<td>Kas</td>
<td>v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog</td>
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<tr>
<td>Lipf</td>
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<tr>
<td>Lsm1</td>
<td>LSM1 homolog, U6 small nuclear RNA associated</td>
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</tr>
<tr>
<td>Msd3</td>
<td>Major facilitator superfamily domain containing 3</td>
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<tr>
<td>Mpv17</td>
<td>Mpv17 transgene, kidney disease mutant-like</td>
<td>Yes</td>
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<tr>
<td>Npelc4</td>
<td>Nuclear protein localization 4 homolog (S. cerevisiae)</td>
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</tr>
<tr>
<td>Nxl1</td>
<td>Nucleoredoxin-like 1</td>
<td>Yes</td>
</tr>
<tr>
<td>Pad3</td>
<td>Peptidyl arginine deiminase, type III</td>
<td>Yes</td>
</tr>
<tr>
<td>Pex16</td>
<td>Peroxisomal biogenesis factor 16</td>
<td>Yes</td>
</tr>
<tr>
<td>Pha2 g2e</td>
<td>Phospholipase A2, group III</td>
<td>Yes</td>
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<tr>
<td>Ptdnd4</td>
<td>Proteasome (prosome, macropain) 26S subunit, nonATPase</td>
<td>Yes</td>
</tr>
<tr>
<td>Rcl1</td>
<td>RNA terminal phosphate cyclase-like 1</td>
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<td>Reep6</td>
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<tr>
<td>Rf146</td>
<td>Ring finger protein 146</td>
<td>Yes</td>
</tr>
<tr>
<td>Rora</td>
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</tr>
<tr>
<td>Scl9a6</td>
<td>Solute carrier family 9 member 6</td>
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<td>Sphk2</td>
<td>Sphingosine kinase 2</td>
<td>Yes</td>
</tr>
<tr>
<td>Tbata</td>
<td>Thymus, brain and testes associated</td>
<td>Yes</td>
</tr>
<tr>
<td>Tinag</td>
<td>Tubulointerstitial nephritis antigen</td>
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<td>Tneml20a</td>
<td>Transmembrane protein 120A</td>
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<td>Trfr2</td>
<td>Transferrin receptor 2</td>
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<tr>
<td>Vmm2r1</td>
<td>Vomeronal 2, receptor 2</td>
<td>Yes</td>
</tr>
<tr>
<td>Zwint</td>
<td>ZW10 interactor</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Boldface indicates genes that are within the top 200 most significantly upregulated genes in 6°C versus 28°C subcutaneous WAT.

Fig. 7. Motif analysis of brite gene promoters. Motif logos are presented from the MEME-LaB-analyzed gene cluster for transcripts induced by cold in subcutaneous WAT and more highly expressed in BAT vs. WAT depots. The MEME-LaB software determined 10 motifs, and these were analyzed for similarity to known motifs from JASPAR, PLACE, and TRANSFAC using position-specific scoring matrix (PSSM). Eukaryotic transcription factor binding sites are indicated for each relevant PSSM.
clude that NRG4 produced by brown adipocytes in culture can affect the neurite development in neural cells. This indicates that NRG4 represents a novel brown adipose tissue adipokine with important autocrine/paracrine functions in the development of the innervation net in adipose tissues, with implications in the acquisition of BAT features in WAT depots.

**DISCUSSION**

In the present study, we aimed to identify factors, pathways, and regulatory elements that are important for acquiring brown fat features in WAT depots. We conducted global expression analysis on discrete adipose tissue depots, with well-recog-
nized differential physiology and involvement in metabolic disease, in mice that had been exposed to cold for 10 days. This stimulus drives the appearance of brown adipocytes or the browning of determined WAT depots. Our results support previous findings that the subcutaneous adipose depot is most reactive to acquiring BAT characteristics, whereas visceral depots are much less responsive (28). Female gonadal WAT shows a certain degree of induced expression of UCPI, PGC-1α, Cidea, and other BAT-specific genes upon adrenergic activation triggered by cold exposure. Mesenteric WAT seems to be almost completely insensitive at the level of gene expression, although interestingly, adipocytes here become multilocular, albeit UCP1-ve, in response to cold (40). In contrast, BAT shows the highest number of genes that are affected upon cold exposure. We have also identified a set of genes and pathways that are either induced or repressed by the tissue remodeling that occurs after the long-term adrenergic activation induced by cold exposure. VEGF signaling was identified as downregulated by cold exposure, with Vegfc specifically reduced and also more highly expressed in WAT compared with BAT. This contrasts with Vegfa, which was found to be present at higher levels in BAT. This agrees with previous reports that associate Vegfa (64) with angiogenesis in BAT and Vegfc being negatively regulated by adrenergic signaling (5) and indicates distinct roles for Vegfc and Vegfa signaling in white and brown adipocytes, respectively.

We have highlighted a set of genes, the expression of which can be induced by these signaling events. These include Bmp8b, a member of the TGFβ family that has recently been found to be involved in brown adipose tissue activation (62); Nrg4, a member of the neuregulin family involved in neurite growth (23); GPR120, a GPCR that has been shown to mediate the anti-inflammatory and insulin-sensing effects of ω-3 fatty acids, with its absence making mice prone to obesity (29); Rgs7, a regulator of G protein signaling that can inhibit signal transduction by increasing the GTPase activity of G protein-α subunits (4); Slc27a2, an isozyme of the long-chain fatty acid-coenzyme A ligase family that has the capacity to convert free long-chain fatty acids into fatty acyl-CoA esters and also function as a fatty acid transporter (26); Letm1 a mitochondrial protein involved in calcium transport (60); and Nnat, a member of the proteolipid family of amphipathic polypeptides that exhibits paternally inherited imprint expression and is considered to play an important role in brain development (33) and glucose-mediated insulin secretion (32). For some, this is the first time that expression has been described in adipocytes. More importantly, with our strategy, we have been able to identify genes that have implications in metabolic pathophysiology in mouse and/or humans, including GPR120 (29), Rgs7 (2), and Nnat (58), that have been reported to be associated with human obesity in linkage and single-nucleotide polymorphism analysis. Further studies will address the relevance of these molecules for adipocyte/adipose tissue functionality.

We have compared the transcriptional differences in our microarray with other available data sets. In the present study, because intact females were used, some genes may be subject to cyclical hormonal changes. Although there were a number of technical differences between studies, including using the C57BL/6 strain, the use of males, and maintenance at 23°C [GEO accession no. GSE44059 (44)], the fact that the same microarray platform was used and expression values were available for exactly the same probes allowed us to perform a direct comparison of the differentially expressed transcripts. This was conducted in OrderedList, a package in R, for meta-analysis of ordered gene lists like those acquired from differential gene expression analysis (37). The P value for a significant overlap between the two expression studies being substantially different from random studies is <10^-10. Nrg4, Slc27a2, Rgs7, and Nnat were differentially expressed in the GSE44059 data sets for BAT vs. inguinal WAT and BAT vs. epididymal WAT. The same genes were also differentially regulated in an independent microarray of BAT vs. epididymal WAT [GEO accession no. GSE8044 (48)]. Our experiments were performed at thermoneutrality, which may reduce the baseline expression of BAT genes in WAT, thereby allowing the identification of additional cold-regulated genes such as GPR120 and Bmp8b, which were not differentially expressed in the other data sets.

In contrast to BAT-enriched genes, the data sets are remarkably divergent for genes that are more highly expressed in WAT compared with BAT. Upk3b showed the greatest difference in expression between BAT and Mes WAT in our study at 28°C and was found in GSE44059 (BAT vs. epididymal WAT) and GSE8044 (BAT vs. subcutaneous WAT). Similarly, Nnat was also found in these analyses. Other genes consistently elevated in WAT compared with BAT across the different data sets include Sneg, Ighv14-2, Ildr2, Samsn1, and Synpo2.

With regard to other analyses of cold-dependent changes in gene expression in inguinal WAT, the genes we have identified are highly consistent with a comparison of a 5-wk exposure to 4 vs. 30°C male C57BL/6 mice [GEO accession GSE13432 no. (64)]. Importantly, Nrg4, GPR120, Slc27a2, Rgs7, Letm1, Bmp8b, and Cil6l (9030619P08Rik) were also cold induced in this microarray. In contrast, there was little consistency in the genes more highly expressed at 30 vs. 4°C in the independent experiments. It is noteworthy that Nnat was one of the few genes that was found in both microarrays (Acn1, Ccr2, Far1, Pida3, Zfp36l, Tnfrsf21, and Ostc were also more highly expressed at thermoneutrality in the 2 analyses).

The consistency across our microarray, GSE44059, GSE8044, GSE13432, and other published studies confirms the expression profile of already identified BAT and brite genes implicated in transcriptional regulation (Zic1, Lhx8, PGC-1α, and Prdm16), mitochondrial function (Cox7a1, UCP1), lipid metabolism (Gyk, Fabp3, Pin5, Elovl3, Park1, and Cidea), cytokine signaling (Otop1), enzymatic activity (Dio2, Cpn2), and coagulation regulation (Kng1).

It is transcriptional regulators that will be pivotal to expression of the brown fat phenotype. Our analysis of relevant controls showed PGC-1α and PRDM16 were more highly expressed in BAT compared with WAT depots, and the subcutaneous WAT depot showed increased levels of both factors in response to cold exposure. Therefore, these transcriptional regulators or their interplay with coregulators such as RIP140, which at 4°C was lower in BAT vs. WAT, may be central to the changes in gene expression due to cold or the depot-specific profiles. Further work is necessary to determine the role of other factors such as Zic1 and Lhx8 in brown and brite gene expression.

We sought to identify regulatory motifs that were specifically enriched in the promoters of the cluster of brite genes
found from the microarray. Analysis using the MEME-LaB software determined the association of Sp1-binding sites with one of the motifs. This transcription factor may be relevant to induction of BAT genes due its interaction with PPARγ (1, 52). Other notable transcription factors that had putative binding sites enriched in the promotors of the brite cluster include p53 and C/EBPβ, which are known regulators of BAT differentiation (35, 38). HNF-4 has recently been reported to be downregulated in WAT by cold, and therefore, it may serve to repress BAT gene expression in this tissue (50). Other factors with putative binding sites, such as BCL-6, SFI, Pax6, and ATF-5, showed elevated expression in BAT compared with WAT, and therefore, they represent novel potential regulators of the brown or brite gene expression pattern.

It is well established that adipose tissues are composed of lipid-filled mature adipocytes and other nonadipocyte cells, which form the SVF. The SVF consists of various types of cells, including immune cells, fibroblasts, pericytes, endothelial cells, adipocyte precursors, and stromal cells as well as undefined pools of stem cells. Fractionation of the different depots showed that all of the genes that were selected from the microarray for validation or further study were enriched in adipocytes compared with the SVF. To evaluate isolated adipocytes from discrete depots, we generated cell lines from precursors of four different adipose depots (BAT and subcutaneous, gonadal, and mesenteric WAT). Our immortalization protocol, which involves the inactivation by temperature of the SV40 T antigen prior to differentiation, prevents sequestration of retinoblastoma protein, avoiding the risk of driving white preadipocytes into “brown-like” adipocytes (45). The adipocyte cell lines demonstrate clear characteristics that correspond to their depot of origin, with the highest expression of UCP1, Cidea, Rgs7, and Nrg4 detected in the differentiated brown adipocytes. Cil6l was present at the highest levels in differentiated subcutaneous white adipocytes, in agreement with tissue expression pattern. The induction of UCP1 and PGC-1α, in response to a β3-stimulus, was greatest in the adipocytes generated from BAT, followed by subcutaneous WAT and gonadal WAT, with mesenteric WAT being unresponsive. The cell lines we generated retain functional β-adrenergic and insulin pathways, oxidative metabolism, and an inflammatory response that very similar to what is found in the different adipose tissue depots. Overall, we can conclude that these cell lines are a reliable model for in vitro studies of different adipose tissue depots and are a powerful tool for future drug discovery studies.

We undertook further study of Nrg4, as it was both highly enriched in BAT vs. WAT and upregulated in WAT following cold exposure and seemed likely to be an important novel signaling factor in adipose tissue that could have a key role in adipocyte-neuronal cross-talk. We found Nrg4 to be secreted by brown adipocytes but not preadipocytes. Using PC12 pheochromocytoma cells stably expressing the receptor for NRG4 and ERBB4, we have been able to show that NRG4 is a BAT/brite adipokine. One of the key differences between brown and white adipose tissue is the degree of sympathetic innervation (54). Previous studies have indicated that nerve growth factor is produced in BAT (41). Our data indicate that NRG4 could be a key factor for the development of BAT and acquisition of BAT features of WAT by promoting the growth of neurites and thus enhancing innervation of the tissue needed to activate the thermogenic functions. To fully evaluate the role of Nrg4 in the regulation of adipose tissue innervation, further studies will require the generation of mouse models with depot-specific ablation of the gene.

The discovery that adult humans possess functional BAT has opened the possibility for future treatments against obesity that would boost BAT activity or promote the appearance of brown adipocytes in nonclassical BAT locations where white fat predominates by BAT-inducing drugs or other therapies, such as tissue transplantation or gene therapy. The identification of the key transcriptional events associated with browning of WAT is important for the understanding of how gene expression governs this tissue remodeling.

GRANTS

This work was supported by Biotechnology and Biological Sciences Research Council Grant BB/H020233/1, the EU FP7 project DIABAT (HEALTH-F2-2011-278373), Wellcome Trust Grant WT093082MA, and the Genesis Research Trust.

DISCLOSURES

The authors declare that they have no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS


REFERENCES


