Recruited vs. nonrecruited molecular signatures of brown, “brite,” and white adipose tissues

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THE LAST FEW YEARS HAVE SEEN A PARADIGM SHIFT with regard to the understanding of the origin of and relationship between adipose tissues. A series of investigations have demonstrated that brown and white adipocytes are not sister cells, but rather, brown adipocytes are closely related to myocytes, and both originate from a common “adipomyocyte” precursor (3, 32, 37). Furthermore, even among classical white adipocytes, it would seem that two types exist: the “genuine” white adipocytes and also “brite” adipocytes, a type of adipocyte that possesses the ability to express the uncoupling protein 1 (UCP1), which until recently was believed to be a unique marker for brown adipocytes (17, 29, 32). Although differences in gene expression pattern were generally quantitative, some gene markers showed, even in vivo, remarkable depot specificities: Zic1 for the classical BAT depots, Hoxc9 for the brite depots, Hoxc8 for the brite and white in contrast to the brown, and Tcf21 for the white depots. The effect of physiologically induced recruitment of thermogenic function (cold acclimation) on the expression pattern of the genes was quantified; in general, the depot pattern dominated over the recruitment effects. The significance of the gene expression patterns for classifying the depots and for understanding the developmental background of the depots is discussed, as are the possible regulatory functions of the genes.

uncoupling protein 1; PR domain containing 16; sirtuins; short stature homeobox 2; carbonic anhydrase 3

Waldén TB, Hansen IR, Timmons JA, Cannon B, Nedergaard J. Recruited vs. nonrecruited molecular signatures of brown, “brite,” and white adipose tissues. Am J Physiol Endocrinol Metab 302: E19–E31, 2012. First published August 9, 2011; doi:10.1152/ajpendo.00249.2011.—Mainly from cell culture studies, a series of genes that have been suggested to be characteristic of different types of adipocytes have been identified. Here we have examined gene expression patterns in nine defined adipose depots: interscapular BAT, cervical BAT, axillary BAT, mediastinic BAT, cardiac WAT, inguinal WAT, retroperitoneal WAT, mesenteric WAT, and epididymal WAT. We found that each depot displayed a distinct gene expression fingerprint but that three major types of depots were identifiable: the brown, the brite, and the white. Although differences in gene expression pattern were generally quantitative, some gene markers showed, even in vivo, remarkable depot specificities: Zic1 for the classical BAT depots, Hoxc9 for the brite depots, Hoxc8 for the brite and white in contrast to the brown, and Tcf21 for the white depots. The effect of physiologically induced recruitment of thermogenic function (cold acclimation) on the expression pattern of the genes was quantified; in general, the depot pattern dominated over the recruitment effects. The significance of the gene expression patterns for classifying the depots and for understanding the developmental background of the depots is discussed, as are the possible regulatory functions of the genes.

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MATERIALS AND METHODS

Sampling of Tissues

The experiments were approved by the Animal Ethics Committee of North Stockholm. To avoid problems associated with differential gene expressions in inbred strains such as C57Bl/6 (17), and in continuation of our earlier studies, we used outbred Naval Medical Research Institute (NMRI) mice here. Male NMRI mice (6 wk old) obtained from a local supplier (B & K, Stockholm, Sweden) were housed at room temperature for ≥48 h after arrival and were then divided into two groups; one was transferred to 30°C and one to 4°C. Both groups had free access to food (chow: R70 Lactamin) and water. After 3 wk at the acclimation temperatures, the mice were euthanized, and samples from the brown and white adipose tissue depots (and muscle) detailed below were dissected out and immediately frozen in liquid nitrogen. The samples were taken from the central parts of each depot.

Adipose Tissue Depots Identified and Examined

Based on investigations by us (29, 37, 41) and others (10, 13, 36, 38, 39, 43), a number of expressed genes that are suggested to characterize (or possibly determine) the different adipocyte lineages have been identified. However, most of the characterization of these markers has been performed in adipocyte cell cultures or in only a limited number of adipose tissues. Although there are clearly interpretation advantages of studying cell cultures, the underlying question must with time be approached: to which degree can the conclusions from the in vitro studies be extrapolated to in vivo conditions?

Therefore, in the present investigation, we have undertaken to examine a broad spectrum of the definable adipose depots of mice to extend the information concerning cell lineage markers to the physiology of the intact animal, allowing for the regulatory systems of the intact animal to influence the development of the adipose cells. Particularly, we have contrasted conditions where the brown and potentially “brite” adipose depots would be minimally stimulated (i.e., the mice are maintained at thermoneutrality and on a chow diet) with conditions where these tissue depots would be maximally adrenergically stimulated, i.e., in mammals in the maximally recruited state: mice that have been acclimated to cold for more than 3 wk.

We found that each adipose depot examined was characterized by a unique marker gene expression pattern. However, despite this, we conclude that it is possible to divide the depots into three main types, the classical brown adipose tissue (BAT) depots, the brite adipose tissue depots, and the (“genuine”) white adipose tissue (WAT) depots, and to associate particular gene expressions patterns with these depots.

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neck. This depot is the deep cervical since there is also a superficial cervical depot conjoint with the iBAT (6).

AXILLARY BAT. Axillary BAT (aBAT) is an intermuscular depot (~100 mg) found directly beneath the scapulae (shoulder blades).

MEDIASTINIC BAT. Mediastinic BAT (mBAT) is an intrathoracic depot (~10 mg) found in the mediastinum. The largest mass of the depot, which is used in this study, is in the arch of the aorta.

Mammals may have intercostal BAT depots (in between the ribs) (4); however, we could not identify such depots in mice.

PERIRENAL BAT. Perirenal BAT (prBAT) is a depot embedding the major part of the renal hilum of the kidney, where the renal artery enters and the ureter and renal vein exit the kidney. We did not include this depot in this study.

Whitish depots. CARDIAC WAT. Cardiac WAT (cWAT) is a thin visceral white depot (~10 mg) lining the left ventricle of the heart down to the apex.

INGUINAL WAT. Inguinal WAT (iWAT) is often understood as the depot attached dorsally along the pelvis and skewed ventrally down on to the thigh of the hindlimb. This depot is routinely considered to represent subcutaneous adipose tissue in mice and is the largest subcutaneous depot (~500 mg). This depot is also described as flank subcutaneous WAT (43) or posterior subcutaneous fat (divided into the dorsolumbar, inguinal, and gluteal portion) (6). The dorsolumbar portion can be regarded as dorsolumbar WAT; however, it is often included together with the inguinal portion since there is no way to separate the two by eye (only in cold-acclimated mice does the dorsolumbar portion acquire a brown color, whereas the inguinal portion is much lighter brown). The third portion is the gluteal portion, which is a small depot close to the reproductive organs and close to but seemingly separated by a gap from the inguinal portion, potentially regarded as gluteal white adipose tissue. In this study, we consistently sampled the central portion. Therefore, the dorsolumbar and the gluteal portions were not included in this study.

RETROPERITONEAL WAT. Retroperitoneal WAT (rWAT) is a depot (~200 mg) attached on the dorsal wall of the back, encapsulated in a thin membrane. This depot is not attached to the kidney or entangled with prBAT.

MESENTERIC WAT. Mesenteric WAT (mWAT) is a visceral depot (~500 mg) found embedded in the mesenterium lining the surface of the intestines.

EPIDIDYMAL WAT. Epididymal WAT (eWAT) is the largest visceral depot (~700 mg) in male mice. This depot embeds the vas deferens, testicular arteries, and the epididymus. Most reports discussing visceral adipose tissue in (male) mice refer to this depot. Due to the presence of the vas deferens, this depot may be sensitive to contamination with tubular genes. Here we have only included the top portion of the depot.

bsWAT, bsWAT is the interscapular whitish adipose tissue found on top of iBAT (43). We found it difficult to include it as a genuine subcutaneous adipose tissue depot, since it is entangled with the interscapular BAT.

ANTERIOR SUBCUTANEOUS WAT. Anterior subcutaneous WAT (asWAT) is the bilateral superficial subcutaneous adipose tissue between the skin and muscle fascia posterior to the lower segment of the upper limbs (6, 33). We did not include this depot in this study.

OMENTAL WAT. Omental WAT (owAT) is a visceral intra-abdominal depot visualized as a thin fat depot lined across the surface of the stomach that is not frequently investigated in mouse studies (26). We were not able to identify this depot, since young mice (9 wk) were used in this study.

We have not sampled the subdermal adipose tissue depots (the thin layer of fat on the inside of the skin) or intramuscular adipose tissue depots found within muscle bundles. As a representative muscle sample, we used the gastronomic muscle, with the gastrocnemius muscle from the hind leg.

RNA Isolation and Real-Time qPCR

Total RNA was extracted from frozen adipose tissue with Ultraspex (Biotex, Houston, TX) according to the manufacturer’s protocol, and RNA concentrations were measured on a Nanodrop nd-1000 spectrophotometer (Thermo-Scientific, Wilmington, DE). To synthesize cDNA, 200 ng RNA from each sample were reverse-transcribed with a High Capacity cDNA kit (Applied Biosystems, Foster City, CA) in a total volume of 10 μl and diluted to 200 μl. To measure each gene of interest (GOI), 4 μl of each cDNA sample was loaded in duplicate in SybrGreen Jumpstart Taq Ready-mix (Sigma-Aldrich) together with prevalidated, designed primers (Table 1) obtained by using the Universal Probe Library online site (Roche Applied Science) on a 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA). All mRNA levels were normalized to transcription factor IIB (TFIIB) mRNA according to the comparative threshold method (Ct method) (ΔΔCt). The equation 2−ΔΔCt was applied to convert the logarithmic ΔΔCt values to linear values.

For miR-206 microRNA measurements, total RNA was diluted to 5 ng/μl. In the microRNA-cDNA synthesis, 2.5 μl of these dilutions was reverse-transcribed in 3.5 μl of reaction mix and 1.5 μl of the miR-206-specific reverse-transcription primer (Table 1) provided with the MicroRNA Assay kit (Applied Biosystems); 1.2 μl of miRNA-specific cDNA from this reaction was amplified in duplicate with the TaqMan Gene Expression mastermix and the probe/primer mix provided in the MicroRNA Assay kit (Applied Biosystems) in the TaqMan qPCR system, as described above. TFIIB mRNA was used as an endogenous control; log- to linear-transformed microRNA/TFIIB...
RESULTS

mRNA expression ratios were calculated as described above for the GOI mRNA.

Reference Gene

As is customary in gene expression studies, all mRNA levels have been expressed here in relation to a reference gene expected to be equally expressed in all cells under all circumstances. The choice of reference gene is not trivial, because such genes may not be as stable in their expression as anticipated either between tissues or between different physiological conditions. We have chosen TFIIB as a reference gene. In Fig. 2, we have examined to which extent TFIIB fulfills the minimal requirement for being a reference gene in the present investigation. We observed that the expression level of TFIIB was fairly similar between the depots and was not markedly altered due to chronic cold.

In addition to the results presented here, an initial experiment performed on a number of adipose depots from another mouse strain (FVB/N) showed results qualitatively similar to those shown here.

Table 1. Primer sequences and microRNA assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>Ca3</td>
<td>GCTCTCGTAAGACGATCC</td>
<td>ATTCGGGAAGCTCGTCC</td>
</tr>
<tr>
<td>Dpt</td>
<td>GTCGCCGCTATAAGAAGGT</td>
<td>TGCTGGATCTCTGTTG</td>
</tr>
<tr>
<td>Hox8</td>
<td>GTCCTGCAGGCTCTGTTTTC</td>
<td>TCCGGTGCAGGCCCGTT</td>
</tr>
<tr>
<td>Hox9</td>
<td>GAGCAAAAGCAGAAGGAAAG</td>
<td>GGGTCTGACGTGTCAG</td>
</tr>
<tr>
<td>Igfbp3</td>
<td>GAGAGCTGACGACCTACCTCC</td>
<td>TCCCTGGAGTACGAGT</td>
</tr>
<tr>
<td>Inhbb</td>
<td>CGAGATCATCAGCTTTGAG</td>
<td>GTGGTCCTCATTAGAGCA</td>
</tr>
<tr>
<td>Lhx8</td>
<td>GAGCTGCGAGCAGCTTCCA</td>
<td>TGGTTCTGAGCAGGAGGCT</td>
</tr>
<tr>
<td>Meox2</td>
<td>CTTTGACGCGGCTTCAATT</td>
<td>AACCTGAGCAGGAGGCT</td>
</tr>
<tr>
<td>Mylfp</td>
<td>TGACGAATGGGCGGAAAAAG</td>
<td>GGGTACGAGGAGGCT</td>
</tr>
<tr>
<td>Prdm16</td>
<td>CAGACGGGTAAGGACATTCC</td>
<td>GGGTACGAGGAGGCT</td>
</tr>
<tr>
<td>Shox2</td>
<td>TGAGAAAATCTACGAGCAGGAG</td>
<td>TTCAAAATCTTCGAGGCT</td>
</tr>
<tr>
<td>Sirt1</td>
<td>TCGTGGAGACTTTTCTAAGGG</td>
<td>GGGTCTGAGGAGGCT</td>
</tr>
<tr>
<td>Sirt3</td>
<td>TGCCTGTTAAACAAGGAGGAT</td>
<td>TCCCAACAGAGGATAGG</td>
</tr>
<tr>
<td>Sirt5</td>
<td>CGGCCTGACGAGCAAAAGG</td>
<td>GACCTGATGTCAGGAG</td>
</tr>
<tr>
<td>Tbx15</td>
<td>CGAATGATCGAGGAGGATG</td>
<td>ATGGCAGAAACATCTAGCT</td>
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<tr>
<td>Tcf21</td>
<td>CATTGGACAGGAGGAGAGTG</td>
<td>TCGTGGAGATGATCTG</td>
</tr>
<tr>
<td>TFIIB</td>
<td>TGAGAATGTTGCGAGGAGAG</td>
<td>GAATTCGAGGAGGCTC</td>
</tr>
<tr>
<td>UCP1</td>
<td>GGCTCCTGACGCTTCACTCA</td>
<td>TAAAGCCGCTGACGTT</td>
</tr>
<tr>
<td>Zic1</td>
<td>AACCTGAGTACCCCAAAAAAGGA</td>
<td>CCTGCGAGCTGACGTT</td>
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<td>hsa-microRNA-206</td>
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<td>000510</td>
</tr>
</tbody>
</table>

Ca3, Carbonic anhydrase 3; Dpt, dermoprotein; HOx8 and Hox9, homeobox C8 and C9, respectively; Igfbp3, IGF-binding protein-3; Inhbb, inhibin; Lhx8, lim homeobox 8; Meox2, mesenchyme homeobox 2; Mylfp, myosin regulatory light chain; Prdm16, PR domain containing 16; Shox2, short stature homeobox 2; Sirt1, -3, and 5, sirtuin 1, 3, and 5, respectively; Tbx15, T-box 15; Tcf21, transcription factor 21; TFIIB, transcription factor IIB; UCP1, uncoupling protein 1; Zic1, zinc fingers in the cerebellum 1. Quantitative PCR primer sequences. Before the measurements, the intron-spanning primer pairs for each mRNA, designed and obtained from the Universal Probe Library, were validated by a standard curve to verify the amplification efficiency and the linear range of amplification (not shown). The mRNA assay from Applied Biosystems consisted of a miRNA-specific RT primer and a miRNA-specific quantitative PCR probe-primer mix.

Fig. 2. Expression levels of the transcription factor IIB (TFIIB) used here as reference gene. Data are means ± SE from 6 mice in each group. Note that the absolute levels of TFIIB mRNA are very similar in the different tissues and that cold acclimation in no case led to a significant alteration in level.

neous, and the samples will not consist purely of parenchymal cells; rather, other cell types will unavoidably be present, including endothelial cells, blood cells, and cells from the immune system, etc. Additionally, even the parenchymal cells may be in different phases of development. Nonetheless, mature parenchymal cells should contribute a significant part.

With real-time PCR techniques, extremely low levels of mRNAs can be detected. Therefore, we find it of importance to present gene expression data in semi-absolute terms. Therefore, all data are expressed as the number of mRNAs of the respective genes of interest, and thus the reference gene chosen compensated for these variations.
Under thermoneutral conditions, UCP1 was well expressed in BAT and considered as being associated with BAT, UCP1 and tissue depots, we examined the expression levels of two genes: UCP1 and Prdm16.

**Gene Expression**

A Classification of Adipose Tissues Based on Innate UCP1 Gene Expression

To make an initial classification of the different adipose tissue depots, we examined the expression levels of two genes respectively chosen primarily from those that we demonstrated earlier (37) to be markers for brown vs. white adipocytes in culture as well as those observed in rosiglitazone-induced white adipocyte cell cultures (“brite adipocytes”) (29). We will start with the most brown-fat-associated markers and progress toward the brite and white markers.

**UCP1**

As expected, UCP1 was not found in muscle (Fig. 3A). Under thermoneutral conditions, UCP1 was well expressed in only four depots of adipose tissue: the interscapular, the cervical, the axillary, and the mediastinal depots. Therefore, we will refer to these depots as BAT depots: iBAT, cBAT, aBAT, and mBAT. Acclimation to cold led to highly significant increases in the expression levels in these depots. However, the increase was only about twofold (Fig. 3A). This twofold difference may be considered less than expected but is in accordance with earlier observations under similar conditions (21); UCP1 mRNA levels in iBAT, expressed relative to total RNA, increase fivefold during the first days in the cold but then successively return toward initial levels. However, cold acclimation is also associated with a approximately threefold higher total RNA amount in the total tissue depot compared with thermoneutrality, leading to a totally ≈10-fold higher UCP1 mRNA amount in the BAT of cold-acclimated vs. thermoneutral mice (21). This leads to a total amount of UCP1 protein in the BAT that is 10- to 50-fold higher in cold-acclimated than in thermoneutral mice (2, 21). These studies together thus underline that alterations in UCP1 mRNA levels may be poor indicators of changes in the total capacity for thermogenesis of classical BAT depots based on the tenet that total UCP1 amounts in the tissues determine total thermogenic capacity (5).

In a further three depots of adipose tissue, the cardiac, the inguinal, and the retroperitoneal depots, UCP1 mRNA was practically undetectable under thermoneutral conditions but became fairly well expressed in cold-acclimated mice (Fig. 3A and Table 2). We will refer to these depots that are apparently “white” at thermoneutrality as cWAT, iWAT, and rWAT. We now classify these depots as “brite” depots in that (certain cells within) these tissues have the physiological ability to express UCP1 but do not always do so. That these depots have a potential capacity to express UCP1 under conditions of high adrenergic stimulation has been observed earlier (17, 42, 44), as comprehensively reviewed (18). It should be noted that the relative increases in UCP1 mRNA level are remarkably high in these depots, 12-fold in cWAT, 82-fold in iWAT, and 239-fold in rWAT (Table 2), but this reflects mainly the practical absence of expression in the nonrecruited state. These relative increases are clearly impressive as such, but it is clear from Fig. 3A and Table 2 that even such high increases result only in UCP1 mRNA levels that are still lower than the levels found in unstimulated classical BAT and about an order of magnitude lower than those found in classical BAT under the same conditions.

**Table 2. UCP1 mRNA expression in adipose tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>30°C</th>
<th>4°C</th>
<th>Cold-Induced Increase</th>
<th>Cold Expression Level vs. iBAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>iBAT</td>
<td>85 ± 3.6</td>
<td>268 ± 13</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>cBAT</td>
<td>113 ± 6.2</td>
<td>219 ± 9.2</td>
<td>2</td>
<td>0.82</td>
</tr>
<tr>
<td>aBAT</td>
<td>104 ± 8.8</td>
<td>229 ± 5.5</td>
<td>2</td>
<td>0.85</td>
</tr>
<tr>
<td>mBAT</td>
<td>28 ± 17.0</td>
<td>77 ± 25</td>
<td>3</td>
<td>0.29</td>
</tr>
<tr>
<td>cWAT</td>
<td>3 ± 1.2</td>
<td>36 ± 11</td>
<td>12</td>
<td>0.13</td>
</tr>
<tr>
<td>iWAT</td>
<td>0.69 ± 0.08</td>
<td>57 ± 10</td>
<td>82</td>
<td>0.21</td>
</tr>
<tr>
<td>rWAT</td>
<td>0.07 ± 0.02</td>
<td>16 ± 0.7</td>
<td>239</td>
<td>0.06</td>
</tr>
<tr>
<td>mWAT</td>
<td>0.03 ± 0.005</td>
<td>0.17 ± 0.05</td>
<td>6</td>
<td>0.00063</td>
</tr>
<tr>
<td>cWAT</td>
<td>0.03 ± 0.007</td>
<td>0.21 ± 0.04</td>
<td>7</td>
<td>0.00078</td>
</tr>
</tbody>
</table>

Values are means ± SE. iBAT, interscapular brown adipose tissue (BAT); cBAT, cervical BAT; aBAT, auxiliary BAT; mBAT, mediastinum BAT; cWAT, cardiac white adipose tissue (WAT); iWAT, inguinal WAT; rWAT, retroperitoneal WAT; mWAT, mesenteric WAT; cWAT, epididymal WAT.
Finally, we identified two depots in which no UCP1 appears at all at the figure resolution given here, the mesenteric and the epididymal adipose tissues (Fig. 3A); these tissues will be referred to as WAT (mWAT and eWAT). Given the ability of qPCR to detect small levels of mRNA, there are apparently measurable levels of UCP1 mRNA even in those depots (Table 2), but these expression levels are, even when highest, still 100-fold lower than the innate levels in BAT. Cold-induced increases in epididymal UCP1 mRNA levels have been reported earlier but only as relative increases (20). However, it may be noted that within the adipocyte precursor population that can be harvested from the epididymal depots, adipocytes with “brite” characteristics can be observed (29).

Prdm16 (also known as MEL1) was identified as one of three transcription factors expressed in iBAT but not in eWAT (34). In the tissues examined here, it can be seen that this difference (or at least a difference in expression of more than an order of magnitude) between the examined tissues could be broadened to all brown depots vs. all white depots. In the brite depots, the expression was in between that in the brown and that in the white depots (Fig. 3B). In cell culture experiments, Prdm16 gene expression is not enhanced when differentiation is promoted with a PPARγ agonist (29), and acute norepinephrine stimulation leads to a diminished expression (our unpublished observations) while increasing UCP1 expression (29). The general conclusion from these observations would be that the Prdm16 expression level does not directly relate to the recruitment status of the brown adipocytes.

In the brite tissues, cold acclimation is not associated with a higher level of Prdm16 gene expression (Fig. 3B), despite the recruitment of the brite cells, as seen by the large increase in UCP1 expression (Fig. 3A and Table 2). This again implies that it is not Prdm16 levels as such that determine the degree of brite cells differentiation, although Prdm16 may be necessary for the differentiation process (33).

Prdm16 has been considered to be a master regulator in the bifurcation between the myocyte and the brown adipocyte fates in the developmental pathway of the adipomyocyte, principally turning off the myogenic pathway (7, 8, 32). In addition, Prdm16 is now also considered to be involved in the differentiation of the brite cells, cells that do not originate from the adipomyocyte precursors within the dermomyotome (32, 33). Prdm16 is also expressed in the lung, heart, placenta, and white depots (Fig. 3B). In cell culture experiments, Prdm16 gene expression is not enhanced when differentiation is promoted with a PPARγ agonist (29), and acute norepinephrine stimulation leads to a diminished expression (our unpublished observations) while increasing UCP1 expression (29). The general conclusion from these observations would be that the Prdm16 expression level does not directly relate to the recruitment status of the brown adipocytes.

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![Figure 4](https://example.com/figure4.png) Fig. 4. Expression levels of the myogenic markers myosin regulatory light chain (Mylpf; A) and microRNA 206 (miR-206; B). The bold arrow above muscle indicates the high mRNA/miRNA level in that tissue. For more details, see legend to Fig. 3. *P < 0.05, significant effects of cold acclimation; Student’s unpaired t-test.

![Figure 5](https://example.com/figure5.png) Fig. 5. Expression levels of lineage-specific gene markers that were not depot-specific markers: mesenchyme homeobox 2 (Meox2; A) and IGF-binding protein-3 (Igfbp3; B). For details, see legend to Fig. 3. *P < 0.05, **P < 0.01, and ***P < 0.001, significant effects of cold acclimation; Student’s unpaired t-test.
kidney as well as in leukocytes and bone marrow cells (22), and it is expressed in a broad range of developing tissues (19). Thus, it would seem that Prdm16 has a broad role in the development (and perhaps proliferation) of a series of different cell types, apparently in certain cases, such as brown and brite adipocytes, delineating the developmental pathway a certain cell will embark on.

**Myogenic Factors**

It was our observation of myogenic markers in undifferentiated cultured brown adipocytes (and their absence in undifferentiated cultured white adipocytes) that originally allowed us to suggest that brown and white adipocytes originate from distinct cell lineages (37). The myogenic mRNA markers were just that, i.e., markers; we did not observe products of the muscle-associated genes at the protein level. The expression level of the myogenic markers decreases with advancing differentiation of the brown adipocytes and has disappeared in fully differentiated brown adipocytes. Here we examined to what extent myogenic markers would be observable in vivo in the adipose depots, perhaps reflecting the undifferentiated precursor component in the tissues of adipomyocyte origin.

Myosin regulatory light chain (Mylpf) is a muscle-associated structural gene expressed in cultured brown adipocytes (40). We observed some Mylpf gene expression in all of the BAT depots [in extension of our earlier findings (40)] (Fig. 4A). However, although Mylpf expression was somewhat higher in the BAT depots than in the brite depots, and >10-fold higher than in the white adipose depots, the level was still some 1,000-fold lower than in muscle. Because the expression of the myogenic genes disappears in maturating brown adipocytes,
low levels would be expected in the tissues, but the Mylpf mRNA observed may correspond to the small fraction of undifferentiated brown precursors found in the brown depots. The tendency toward higher expression in brite than in white depots is not readily explainable, because there is evidence that these depots are not of adipomyocyte origin based on cell lineage studies tracing cells that had at some time expressed the myogenic regulatory factor Myf5 (32). We did not measure Myf5 here, since this gene was practically undetectable (i.e., had even lower levels) in iBAT than those discussed above (40).

MicroRNA 206 (miR-206) is a “myomir” (i.e., a miRNA normally associated with muscle cells) that is also expressed in cultured brown adipocytes (but not in white) (29, 41). miR-206 is thus part of the myogenic signature of brown adipocytes. In contrast to what is the case for those myogenic markers that are mRNAs, miR-206 levels do not decline during brown adipocyte differentiation (29, 41), and therefore, they could function potentially as permanent markers of depots of adipomyocyte origin. We found that miR-206 levels were generally somewhat higher in BAT depots than in the other adipose depot but were some 100-fold lower in muscle (Fig. 4B). However, the differences between the adipose depots were not such that miR-206 could be used as an unequivocal marker for depots of adipomyocyte origin. The TaqMan miR-206 reagents yield a substantial miR-206 signal already in myoblasts, and this signal is not diminished when using antagomirs toward miR-206 (unpublished observations).

There was practically no effect of cold acclimation on these muscle-associated genes. Thus, based on myogenic markers, it was possible to discern differences between BATs of adipomyocyte origin and the brite and white depots, but the differences were not of the qualitative character observed in cell cultures (29, 37, 41), and therefore, there is no marker identified that allows for definitive identification of adipose depots with an adipomyocyte origin in the adult mouse.

**Gene Markers That Did Not Show Depot Specificity**

In our characterizations of differential gene expression in brown and white adipocyte cultures, a series of genes could qualitatively be associated with each cell type. However, as detailed below, two of these markers did not demonstrate a corresponding depot specificity in vivo; one of the genes was brown specific in vitro, and one was white specific in vitro.

Mesenchyme homeobox 2 (Meox2; also known as Gax) is well expressed in cultured brown adipocytes but not in cultured white adipocytes (29, 37). However, Meox2 showed no differ-
ential expression between the different adipose depots (Fig. 5A). In cold-acclimated mice, the expression was systematically repressed in all brown and brite depots but was unaffected in white depots. Meox2 is expressed in smooth muscle (16) and suppresses angiogenesis (15, 28). If the expression seen in all adipose depots is primarily from the vascular cells, the repression seen in cold acclimation may be related to the intensive angiogenesis occurring in brown and brite depots during cold acclimation (42). The reason why brown adipocytes express Meox2 in culture, whereas white adipocytes do not, is not clear but may relate to oxygen sensing, because brown adipocytes are geared genomically to be part of a highly vascular tissue on stimulation.

The Meox genes are essential for development of several tissues; in Meox1/2 double-knockouts, a characteristic feature is the disappearance of both hypaxial muscles and BAT (25), principally in accordance with these tissues originating from the same precursors.

Insulin-like growth factor binding protein-3 is expressed specifically in cultured white preadipocytes and in differentiated white adipocytes (and C2C12 muscle cell lines) (29, 37). However, also for this gene we found no clear depot specificity (Fig. 5B).

Among the genes examined here, which were selected on the basis of earlier in vitro characterizations, only these two genes fully lost their cell lineage characteristic when examined in vivo. There is evidently a possibility that the genes are expressed in cell types other than the depot parenchymal cells and that such cell types dominate the expression.

**Novel Brown Gene Markers That Are BAT Depot Specific**

Lim homebox 8 is expressed specifically in cultured brown adipocytes compared with both white adipocytes and the C2C12 muscle cell line (29, 37). The present analysis showed that it was absent from muscle but well expressed in iBAT and cBAT and less expressed in the other adipose depots. In eWAT it was also absent (Fig. 6A).

Zinc fingers in the cerebellum 1 (Zic1) is expressed specifically in cultured brown adipocytes but is absent in cultured white adipocytes (29, 37). In vivo, Zic1 showed a remarkably distinct expression pattern; it was well expressed in the clas-

cical BAT depots but was totally absent from brite and white adipose depots and was not found in muscle (Fig. 6B). Thus it displayed a brown fat specificity exceeding that of Prdm16 (and even UCP1).

This very distinct expression pattern not only makes Zic1 the marker of choice to identify classical BAT depots but also indicates that Zic1 expression could be important for brown adipocyte differentiation or function. However, there are presently no experiments demonstrating such functions of Zic1.
**A Brown/Brite Depot-Selective Gene Marker**

T-box 15 (Tbx15) is a brown adipocyte marker gene compared with white adipocytes (37). The qualitative distinction observed between the brown and white adipocyte expression in vitro was paralleled here by the presence of Tbx15 in the brown depots (but not in the mediastinal depot) and its total absence in the white depots (Fig. 7). However, Tbx15 was also well expressed in the inguinal brite depot, less so in the retroperitoneal depot, and not in the cardiac depot; all expression levels were much lower than in muscle. Tbx15 levels tended to be repressed by cold acclimation. This observation extends those of Gesta et al. (13), who concluded that Tbx15 was expressed specifically in mouse subcutaneous adipose tissue (apparently the inguinal depot) in contrast to the visceral, intra-abdominal adipose tissue depot (the epididymal depot). Particularly, we find that the expression is not specific for inguinal adipose tissue but is equally high in the brown depots despite the different developmental origin of these depots.

The expression patterns may be said to adhere to the discussed roles of Tbx15 as a repressor of adipogenesis and mitochondriogenesis (12), e.g., in that the level of Tbx15 is decreased during cold acclimation when mitochondriogenesis is activated, although this has not as yet been confirmed in primary cultures.

**Brite Adipocyte Gene Markers**

Two of the examined genes display remarkable brite depot specificity (Fig. 8).

Homeobox C9 (Hoxc9) was described by us originally as white adipocyte specific (29, 37). However, in white adipocyte cultures, Hoxc9 mRNA levels are significantly upregulated by rosiglitazone treatment (that promotes brite adipocytes) (29). Within the adipose tissues, Hoxc9 was found in the brite depots, but it was not increased by cold acclimation, so its expression did not parallel the induction of UCP1 in the brite depots. It was less expressed in white depots. Hoxc9 was not
expressed at all in brown depots, in agreement with Yamamoto et al. (43).
Short stature homeobox 2 (Shox2) has been described as being expressed in flank WAT but not in eWAT (13, 43). Here, we find Shox2 being nearly selectively expressed in iWAT.

White Adipocyte Gene Markers That Are White/Brite Depot Specific

A series of genes were observed to be white adipocyte specific in cell culture (37). These genes include homeobox C8 (Hoxc8), transcription factor 21 (Tcf21), inhibin B (Inhbb), and dermatopontin (Dpt). Here, we examined the expression of these markers in the different tissue depots (Fig. 9, A–D). These markers could broadly be described as being well expressed in the white depots and also well expressed in the brite depots but poorly or not expressed in the brown depots. Cold acclimation led to a reduction in the expression levels of all of these genes. Concerning the white depots, this is remarkable because cold acclimation has not generally been expected to have such effects on genuine WAT depots.

Hoxc8 has been described as being expressed specifically in abdominal (i.e., epididymal) compared with subcutaneous adipose tissue (13). We could not confirm such a qualitative difference (Fig. 9A).

As seen, Tcf21 is the closest we can come to a white fat-specific gene, being highly expressed in eWAT.

Carbonic Anhydrase 3

Carbonic anhydrase 3 (Ca3) is found in BAT (and muscle) (24) and is downregulated in mouse BAT by cold exposure (10). We did not find a tissue-selective expression pattern for this gene, but we observed repression due to cold acclimation specifically in brown and brite depots but not in white depots (Fig. 10). It has been stated that Ca3 protein may constitute 24% of the total protein content of white adipocytes (1). Principally in agreement with this, only Ca3 mRNA levels...
reached the mRNA levels observed for UCP1, i.e., hundreds of mRNAs per TFIIB mRNA. A functional role for this abundant protein has still not been identified.

The Sirtuins

The sirtuins are a family of seven class III histone/protein deacetylases. The sirtuins function as redox sensors by responding to shifts in NAD+/NADH levels. In different contexts, some of the sirtuins have been suggested to be of relevance with respect to adipocyte precursor fate.

Sirtuin 1. Based on a comparison of the altered gene expression caused by sirtuin 1 (Sirt1) overexpression in myoblasts (11) and the altered gene expression occurring during brown adipocyte differentiation (37), we earlier pointed to Sirt1 as a potential regulator in the differentiation of the adipomyocyte precursor into a brown adipocyte (37). Furthermore, Sirt1 could function as a repressor of myogenic factors. However, here we observed similar Sirt1 mRNA levels in all adipose depots as well as in muscle, and the expression did not change as an effect of acclimation to cold (Fig. 11A). Thus, if Sirt1 is involved in repression of the myogenic genes, this effect is not caused by alterations in the mRNA expression level of the Sirt1 gene. In agreement with this, Sirt1 mRNA and protein levels are not well correlated (30). Sirt1 activity may also be regulated posttranslationally (31), and stimulation of Sirt1 by SRT1720 leads to recruitment of BAT (9). Thus, it cannot be excluded that Sirt1 may be involved in the myocyte/brown adipocyte bifurcation.

Sirt3. Sirt3 mRNA levels are high in mature brown adipocytes, in contrast to mature white adipocytes and brown and white preadipocytes (37). We found Sirt3 mRNA to be expressed somewhat higher in brown fat depots than in other adipose depots but found no effect of cold acclimation (Fig. 11B). Sirt3 has been observed earlier to be low in WAT but high in BAT and reported to be increased in response to cold exposure (35) controlled by peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (14). Overexpression of Sirt3 enhances the expression of UCP1, PGC-1α, and mitochondrial genes (35). However, Sirt3-knockout mice have normal energy expenditure and unaltered UCP1 protein levels (23). A specific role for Sirt3 gene expression in brown adipocyte differentiation is not supported by our observations.

Sirt5. Similarly to the case for Sirt3, we found rather similar levels of Sirt5 mRNA in all depots examined, and the expression was not systematically altered by cold acclimation (Fig. 11C). Sirt5 gene expression has been reported elsewhere to be upregulated by cold exposure in BAT (35).

Thus, although sirtuins have been suggested to be involved in the regulation of BAT recruitment, it does not seem that such a regulation is based on regulation of mRNA expression level either between adipose tissues or during cold acclimation. Thus, any involvement of sirtuins in BAT recruitment would have to take place at the posttranscriptional level.

DISCUSSION

In the present study, we have investigated the depot-specific expression of a series of adipocyte markers, identified earlier in in vitro studies as being particularly associated with brown, white, or brite adipocytes, and we have established the effect of recruitment state on these expression levels. Although we found that expression patterns in vivo were not as distinctive as those observed earlier in cell culture systems, we can point to several markers as being particularly informative. All investigated depots present their own unique patterns, but the depots can reasonably be divided into three main types: the brown, the brite, and the white depots. The marker genes characterized here point to separate lineages for these different depots. Understanding the lineages and characteristics of the different depots may advance experimental design in future studies and lead toward an ability to differentially affect different depots therapeutically.

Certain Gene Expression Observations Stand Out

Concerning most of the genes examined here, mainly quantitative differences were observed between the depots. However, a few genes displayed expression patterns with a qualitative difference between depots.

Zic1 gene expression was found to be extremely specific for the three classical brown adipose tissue depots, much more so than Prdm16 and even UCP1 (Fig. 6B). Zic1 was expressed at a reasonably high level (∼1⁄10 the level of Prdm16). These observations would suggest that Zic1 has a functional role in the determination of the classical brown adipocytes, but there are presently no functional studies relating Zic1 expression to brown adipocyte determination, differentiation, or function.

Hoxc9 is remarkably specific for the brite adipose tissue depots, being fully absent from the brown adipose tissue depots (Fig. 8A). Hoxc9 is found both in white and brite adipose depots but is downregulated when the animal is cold acclimated (Fig. 9A). Concerning the homeobox genes, it is not presently possible to distinguish whether the expression of these genes should be seen only as (a memory of) positional markers (which would mean that all white and brite adipose tissue depots may originate from a narrow range of segments.

Table 3. eWAT gene expression vs. iBAT

<table>
<thead>
<tr>
<th>Gene</th>
<th>30°C eWAT vs. iBAT Expression</th>
<th>4°C eWAT vs. iBAT Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zic1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UCP1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tbx15</td>
<td>0</td>
<td>0.04</td>
</tr>
<tr>
<td>Lhx8</td>
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<td>0.005</td>
</tr>
<tr>
<td>miR-206</td>
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<td>0.2</td>
</tr>
<tr>
<td>Mylfp</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Prdm16</td>
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<td>0.05</td>
</tr>
<tr>
<td>Sirt3</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Mox2x</td>
<td>1.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Shox2</td>
<td>1.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Sirt1</td>
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<td>0.7</td>
</tr>
<tr>
<td>Igfbp3</td>
<td>1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Sirt5</td>
<td>2.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Ca3</td>
<td>2.4</td>
<td>28</td>
</tr>
<tr>
<td>Inhbb</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>Dpt</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Hoxc8x</td>
<td>31</td>
<td>23</td>
</tr>
<tr>
<td>Tcf21x</td>
<td>256</td>
<td>240</td>
</tr>
<tr>
<td>Hoxc9x</td>
<td>316</td>
<td>216</td>
</tr>
</tbody>
</table>

Genes are ranked based on expression level in eWAT vs. iBAT at 30°C. *Note that the ratios for these genes arise from division with an infinitely small iBAT value.
during development) or whether they still have functions in
determination of the phenotype of the cells.

Tcf21 is the gene closest to being a selective white adipose
depot marker (Fig. 9B).

The Classification of Depots

A thorough examination of the data presented here clearly
indicates that the initial division into brown, brite, and white
tissues does not imply that the different depots within these
divisions are identical. Concerning each gene examined, differ-
ces in expression level may be discussed. To visualize the
differences between the different depots, in Fig. 12 we have
created tissue fingerprints. These fingerprints were made in
the way that the level of gene expression in iBAT of each gene was
set to 1. The expression in all other depots was expressed in
relation to this. Genes were then ordered on the basis of their
relative expression in the most white adipose tissue, eWAT, vs.
that in iBAT (see Table 3) and depicted (on a logarithmic scale).

As seen in Fig. 12A, in the mature state there is not much
similarity between muscle and brown adipose tissue despite the
fact that they both originate from the same precursors. In the
nonrecruited state, within the traditional brown adipose tissue
depots, the similarity between iBAT and cBAT is very high, and
aBAT is not much different. However, mBAT displays a finger-
print that is quite different from the classical brown adipose tissue
depots but not really skewed toward the eWAT pattern. On the
basis of this and the individual gene expression data, it would
seem that mBAT is distinct from the classical BAT depots.

The expression pattern in all WAT depots in the non-
recruited state is qualitatively different from that of the BAT
depots and is rather similar to the eWAT pattern. When the
BAT depots are examined under recruited conditions where the
tissues have been chronically adrenergically stimulated, they
become more similar in gene expression. Formally, this can be
calculated as a decrease in SE (of the logarithmicized values)
from 0.37 to 0.27 in cBAT and from 0.52 to 0.25 in aBAT [but
only 0.85 to 0.82 in mBAT (Hoxc8 values excluded)]. Even in
the recruited state, the expression profiles of the brite sites are
still closer to the eWAT pattern, demonstrating a maintained
qualitative difference between these depots and the BAT de-

pots. However, these depots, particularly the iWAT, tend to
move toward a brown fat fingerprint in the cold-acclimated
mice (iWAT SE from 1.18 to 1.00).

The white depots were clearly different from the brown, and
there was no tendency that the expression pattern moved
toward the brown in cold-acclimated mice.

Thus, in general, the effects of recruitment were less prom-
inent than the differences between the depot types.

The Relationship Between the Depots

We have used the expression “brite depots” here to func-
tionally identify the depots that express UCP1 in the recruited
state and, e.g., lack Zic1 expression. It is clear that the depots
functionally defined here as “brites,” based primarily on their
ability to express UCP1, include (or are identical with) the
depots that are referred to anatomically as subcutaneous and
that the white depots functionally classified here as those that
do not express UCP1, even in the recruited state, anatomically
include those that are referred to as visceral. It may be noted that
routinely in the literature, observations, e.g., of gene
expression patterns from just one tissue depot are extrapolated
to be valid to all “subcutaneous” or “visceral” depots or to all
brown vs. white depots. It is clear from our data that such
extrapolations may be valid but often are not.

We had observed earlier that about 10% of the preadipocytes
isolated from eWAT from outbred NMRI mice could be
experimentally forced to display UCP1 expression (29). It is
clear that this ability is not displayed in vivo even under strong
recruitment conditions such as those examined here. Thus,
adequate physiological stimuli do not reach this population in
the epidydimal depots (but this does not exclude that pharma-
ceutical agents may have such a “britening” effect even in the
visceral depots). However, in the brite depots, which also lack
the specific BAT markers such as Zic1, physiological stimu-
lulation is sufficient to allow for expression of the brite pheno-
type.

The tenet has been promoted that all adipose tissue depots
should be seen as one organ, i.e., “the adipose organ” (6). It
will be understood that our data are not fully in compliance
with this notion. It seems clear to us that in the mouse there is
a qualitative difference between the various brown adipose
tissue depots and the various white adipose tissue depots. Even
the fact that precursors derived from classical brown (inter-
scapular) and white (epididymal) adipose tissues develop differ-
dently given identical culture conditions (27) point to innate
differences between these tissues. Direct comparative studies
of cells cultured from brite depots compared with white depots
are scarce but would help in delineating which differences
between the depots are cell autonomous and which are second-
ary to local factors being different between depots.

Conclusions

Perhaps to a higher extent than expected, the gene expression
patterns established from studies of adipose cells in primary

culture remain as distinctions when the cells are found in their
natural environment. However, it is clearly so that it is not realistic
to think of adipose tissues as being either brown or white or even
brown, brite, or white. Rather, each depot displays gene expres-
sion patterns that characterize that particular depot and probably
determine its functional character as well.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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