

Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages

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Communicated by Bruce M. Spiegelman, Dana–Farber Cancer Institute, Boston, MA, December 19, 2006 (received for review October 15, 2006)

Attainment of a brown adipocyte cell phenotype in white adipocytes, with their abundant mitochondria and increased energy expenditure potential, is a legitimate strategy for combating obesity. The unique transcriptional regulators of the primary brown adipocyte phenotype are unknown, limiting our ability to promote brown adipogenesis over white. In the present work, we used microarray analysis strategies to study primary preadipocytes, and we made the striking discovery that brown preadipocytes demonstrate a myogenic transcriptional signature, whereas both brown and white primary preadipocytes demonstrate signatures distinct from those found in immortalized adipogenic models. We found a plausible SIRT1-related transcriptional signature during brown adipocyte differentiation that may contribute to silencing the myogenic signature. In contrast to brown preadipocytes or skeletal muscle cells, white preadipocytes express Tcf21, a transcription factor that has been shown to suppress myogenesis and nuclear receptor activity. In addition, we identified a number of developmental genes that are differentially expressed between brown and white preadipocytes and that have recently been implicated in human obesity. The interlinkage between the myocyte and the brown preadipocyte confirms the distinct origin for brown versus white adipose tissue and also represents a plausible explanation as to why brown adipocytes ultimately specialize in lipid catabolism rather than storage, much like oxidative skeletal muscle tissue.

microarray | myocyte | principal component analysis | differentiation | transcriptome

At the onset of obesity, mitochondrial function is attenuated in white adipocytes (1), exemplifying a negative interaction between obesity and mitochondrial biogenesis, a process that potentially contributes to diabetes (2–4). In contrast to white adipocytes, brown adipocytes have an abundance of mitochondria and are able to contribute positively to energy expenditure through the specific expression of the mitochondrial uncoupling protein, UCP1 (5). By using this narrow criterion, primary white adipocytes can also obtain a brown adipocyte-like phenotype (6, 7) through overexpression of peroxisome proliferator-activated receptor γ (PPAR γ) coactivator 1 α (PGC1 α). PPAR γ has been found to be essential for adipogenesis *per se* (8, 9), whereas PPAR γ agonists, such as rosiglitazone, promote reversal of mitochondrial dysfunction in adipocytes (1) and skeletal muscle (3), but unfortunately they also promote adiposity and weight gain, potentially limiting their clinical effectiveness (5).

There is support for the possibility that unique precursor cell types give rise to brown versus white preadipocyte pools because these distinct adipose tissue depots appear at different developmental stages (10). Identification of the transcriptional strategies that characterize the distinct brown adipocyte differentiation process may help identify safe mechanisms to promote mitochondrial

biogenesis or increase energy expenditure in white adipocytes. However, the factors that determine primary adipocyte differentiation remain ill defined (9, 11, 12). We would argue that utilization of a primary cell model is critical to address this research aim because it will faithfully document the endogenous process through which “preadipocytes” pass to form more mature adipocytes. In contrast, immortalized cell models are *a priori* committed to an adipocytic lineage in a manner that may have limited bearing on endogenous differentiation (9), thus potentially limiting their usefulness. In the present study, we contrasted the transcriptional regulation of brown and white primary preadipocytes and robustly demonstrated that brown preadipocytes uniquely share an overlapping transcriptional program with muscle cells, whereas a number of developmental genes further help to distinguish the two preadipocyte cell types.

Results

Discovery of a Myogenic Signature in Brown Preadipocytes. By using a variety of array analysis methodologies we provide here an extensive analysis of primary brown and white preadipocytes early during their respective differentiation programs. Using the Affymetrix U74A v2 chip, we characterized the abundance of $\approx 8,000$ transcripts (12,000 probe sets) in undifferentiated primary brown and white preadipocytes ($n = 6–8$) cultured for 4 days (13). At this stage, the preadipocytes are morphologically undifferentiated yet committed to their final phenotype (see below). By using signifi-

Author contributions: J.A.T., K.W., and O.L. contributed equally to this work; J.A.T., J.N., and B.C. designed research; J.A.T., K.W., O.L., T.B.W., T.L., N.P., D.L.H., R.E.G., and K.B. performed research; J.A.T., K.W., O.L., T.B.W., T.L., D.L.H., R.E.G., K.B., J.N., and B.C. analyzed data; and J.A.T., O.L., C.W., J.N., and B.C. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Abbreviations: bHLH, basic helix–loop–helix; GO, gene ontology; IRS-1, insulin receptor substrate 1; PCA, principal component analysis; PGC1 α , peroxisome proliferator-activated receptor γ coactivator 1 α ; PPAR γ , peroxisome proliferator-activated receptor γ ; RT-qPCR, real-time quantitative PCR; TF, transcription factor.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE 7032).

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This article contains supporting information online at www.pnas.org/cgi/content/full/0610615104/DC1.

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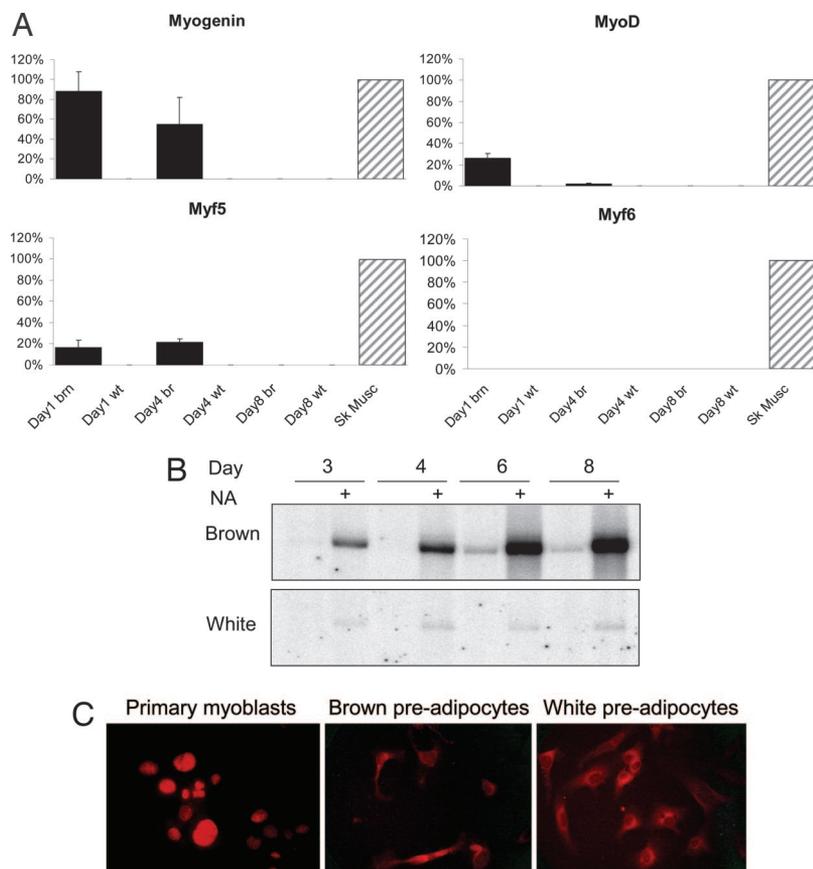


Fig. 1. Phenotypic and myogenic factors in brown and white primary preadipocytes. Cell cultures were generated, harvested at the time points indicated, and examined for expression of genes by using RT-qPCR ($n = 3$ per cell type and time point) or by immunohistochemistry. (A) Myogenin, MyoD, Myf5, and Myf6 mRNA expression was determined by using RT-qPCR. Data (mean \pm SE) are expressed as a percentage of the positive control value (hindlimb muscle obtained from 4-week-old mice). (B) A Northern blot was carried out to demonstrate the *ucp1* expression status in primary white and brown preadipocytes at the time intervals shown. Norepinephrine ($0.1 \mu\text{M}$ for 4 h) was used to induce *ucp1* expression to demonstrate that the brown preadipocytes were committed to the brown cell lineage and distinct from the white preadipocytes. (C) Given the striking findings from the array analysis we aimed to rule out the possibility of any contamination from skeletal muscle cells within the brown preadipocyte culture. Immunohistochemistry was carried out to detect MyoD expression in day 2 preadipocytes (day 2 cultures have enough cells to ensure that a reasonable image can be obtained). Primary mouse myoblasts (Left) demonstrated nuclear staining (red) for MyoD, whereas at the most both brown and white preadipocytes demonstrated diffuse cytosolic staining resulting from the secondary antibody (see SI Fig. 4). At no time did we find any evidence that a distinct subpopulation of cells stained strongly for MyoD, and thus the brown preadipocytes cultures are not contaminated with myoblasts.

cance analysis of microarrays (14) we identified ≈ 300 genes demonstrating at least 2-fold differential expression between brown and white undifferentiated preadipocytes (false discovery rate, $<1\%$) [supporting information (SI) Data Set 1]. The most differentially expressed of these genes are listed in SI Table 1. As can be seen, within the brown preadipocytes “transcriptome” we found a substantial enrichment of genes belonging to the “skeletal muscle” gene ontology (GO) analysis class (15). Remarkably, these genes included the previously claimed “muscle-specific” basic helix–loop–helix (bHLH) myogenic regulator myogenin, which was expressed in brown preadipocytes at a level comparable with that found in differentiating confluent C2C12 myoblasts (16). To confirm the expression of myogenic genes in brown preadipocytes, we used an independent set of primary cell cultures in which we harvested RNA after 1, 4, and 8 days in culture and performed fluorescence-based real-time quantitative PCR (RT-qPCR). Convincingly, in brown preadipocytes (but not in white), myogenin was expressed at day 1 (at a level similar to that in young adult skeletal muscle), with expression diminishing during differentiation (Fig. 1A). Although MyoD and Myf5 were detectable in brown preadipocytes after 1 and 4 days of culture, Myf6 was detectable only in skeletal muscle tissue, demonstrating that brown preadipocytes do not express the full repertoire of myogenic regulatory factors. By using the brown adipocyte marker gene UCP1 (Fig. 1B), we confirmed that despite identical culture conditions, our primary brown cell cultures were phenotypically distinct from our white preadipocytes: increasing norepinephrine-induced *ucp1* induction was only observed in the maturing brown cultures.

Gene expression profiling is a sensitive and powerful tool to study the cellular differentiation process (17), but the issue may be raised that contaminating myoblasts could have influenced the analysis outcome. However, several lines of experimental data and calculation demonstrate that this problem does not exist. For example,

we carefully examined for nuclear staining of MyoD (Fig. 1C) and myogenin (data not shown) in primary myoblasts (18) and contrasted them with HeLa cells artificially contaminated with myoblasts, and with our two primary preadipocyte cell types (white and brown; SI Fig. 4A and B). Primary myoblasts demonstrated clear nuclear staining for MyoD, whereas both brown and white preadipocytes demonstrated only cytosolic staining (a nonspecific action of the secondary antibody; Fig. 1C), a pattern distinct from the artificial “contamination profile” obtained when mixing primary myoblasts with HeLa cells (representative images can be found in SI Fig. 4). The presence in brown preadipocytes of myogenic transcription factor (TF) mRNA is supported by recent findings of Atit *et al.* (19), who published findings clearly demonstrating that the myogenin-expressing central dermomyotome gives rise to dermis, muscle, and brown adipose tissue but not white adipose tissue, data that wholeheartedly support the present analysis. Thus, the lack of MyoD or myogenin protein expression in brown preadipocyte cultures is consistent with the lack of sensitivity of the protein detection methodology and, as one might now expect, remnant myogenic transcription factor (TF) activity reflecting the common developmental origin of brown preadipocytes and striated muscle.

Contrasting Differentiation Processes. We used both correlation and principal component analysis (PCA) (20, 21) to define the differentiation processes in the brown and white adipocyte cultures. Day 4 undifferentiated brown preadipocytes are more similar to maturing brown adipocytes than either to day 4 white preadipocytes or to C2C12 myoblasts (16) (SI Fig. 5A–C). The undifferentiated white preadipocyte transcriptome was specifically enriched with extracellular matrix genes (SI Data Set 1), consistent with a recent cDNA array study examining human preadipocytes (22). There were also evident similarities between the two adipocyte cultures; e.g., *Igf1* and *Pparg* mRNA differed modestly between the cell types

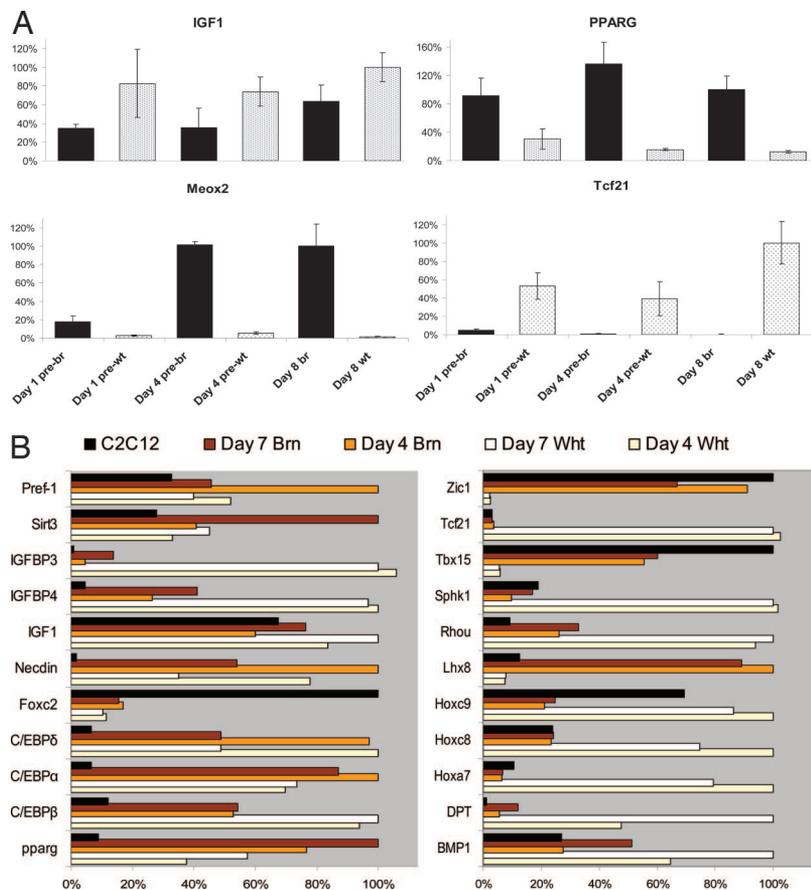


Fig. 2. Expression of established and novel adipocyte marker genes in brown (br) and white (wt) preadipocytes. Primary cultures were produced, and cells were harvested at the time points indicated. (A) RT-qPCR validation of *Pparg* and *Igf1* expression patterns in white (pre-wt) and brown (pre-br) preadipocytes ($n = 3$ independent cultures per cell type). In addition, verification of the microarray data for novel markers for brown (*Meox2*) and white (*Tcf21*) preadipocytes was carried out. (B) Affymetrix hybridization data for literature (Left) and novel (Right) markers of brown and white preadipocytes. Data from C2C12 (see *SI Methods*) are provided as a reference and further illustrate the difference in profile among brown, white, and muscle cells. The analysis is illustrative of genes that are expressed differentially across these cell types. Values are normalized to the cell type with the greatest signal; values much $<20\%$ are indicative of an “absent” call within the MAS5.0 algorithm. Of the “literature” genes, only *Igfbp3* displays a qualitative difference in expression pattern; among the novel genes, *Lhx8* (and *Zic1*) are characteristic of brown adipocytes, and *Tcf21*, *Sphk1*, *Hoxa7*, and *DPT* are characteristic of white adipocytes.

(Fig. 2A). However, to identify potential “unique” markers for brown and white preadipocytes, we examined the array hybridization data by using a simple threshold filter approach (Fig. 2B). Several genes from this analysis appeared interesting, and two were validated further by using RT-qPCR: *Meox2* and *Tcf21*. The vascular developmental gene *Meox2* (mesenchyme homeobox 2) was expressed markedly in brown preadipocytes (Fig. 2A), although it was essentially absent in white adipocytes (Fig. 2A) and in skeletal muscle cells (data not shown). During active thermogenesis, brown adipose tissue becomes highly vascularized (5), and interestingly, *Meox2* can promote VEGF-driven vessel maturation (23), a process central to brown adipose tissue angiogenesis and one that may be distinct (24) from the hypoxia-driven process in skeletal muscle (25, 26). Intriguingly, the bHLH “antimyogenic” transcription factor *Tcf21* (27) (also known as *Pod-1*) appeared distinctly expressed in white preadipocytes (Fig. 2 and *SI Data Set 1*).

We identified the biological processes that were altered during differentiation by examining the genes expressed differentially (28) at day 8 versus day 4 in culture. We applied gene ontology analysis (15) to the $\approx 1,400$ modulated genes in brown preadipocytes and the ≈ 450 modulated in white preadipocytes during maturation (*SI Data Set 2*). Because both cell types become lipid-laden during the differentiation process, it was not surprising that 72% of the genes up-regulated in white preadipocytes were also up-regulated in brown preadipocytes and that 52% of the genes down-regulated in white preadipocytes were also down-regulated in brown preadipocytes (Fig. 3A and *SI Data Set 3*). In both brown and white preadipocytes, extracellular matrix modeling genes were abundantly up-regulated during differentiation, giving rise to the highest ranked enriched group in the gene ontology analysis (Fig. 3A and *SI Data Set 2*). Down-regulated processes included cell cycle and nucleic acid biosynthesis genes (Fig. 3A and *SI Data Set 2*), as

expected during growth arrest, a process known to be essential for adipocyte differentiation (29).

However, there were also substantial differences in the expression programs of the two cell types. For example, only brown preadipocytes substantially switched on an oxidative phosphorylation program, where ≈ 100 mitochondria-related genes were up-regulated (*SI Data Set 2*). Gene ontology analysis demonstrated that this characteristic was unique among brown preadipocytes during differentiation and verified that mitochondrial genes were indeed statistically overrepresented ($P < 0.00001$, Bonferroni-adjusted; *SI Data Set 3*). The most down-regulated genes in brown preadipocytes were myogenin and several other skeletal muscle-related genes (although they represented only a small proportion of the modulated gene list and thus were not detected by gene ontology analysis).

Contrasting Transcriptional Profiles in Primary Preadipocytes and Immortalized Cell Line Models. Studies of an immortalized brown adipocyte cell line have implied that insulin receptor substrate 1 (IRS-1) deficiency (and ensuing necdin overexpression) prevent differentiation of “brown-like” preadipocytes (11). We used the multiple-array study comparison approach (30), comparing our data set and the IRS-1-knockout immortalized brown adipocyte cell line data set (11), to examine consistency between the two models for brown adipocyte differentiation (i.e., we wanted to identify a number of inversely related gene expression responses across these “differentiation” transcriptomes) (*SI Data Set 4*). However, with a 2-fold differential expression data set, we found that only four genes were modulated consistently in the IRS-1 model and our brown adipocyte differentiation model, one of them being *Dkk3*, a wnt signaling “antagonist” that was 2-fold overexpressed in IRS-1 $^{-/-}$ adipocytes cell lines and 2-fold down-regulated during differentiation of brown preadipocytes. Given our ability to

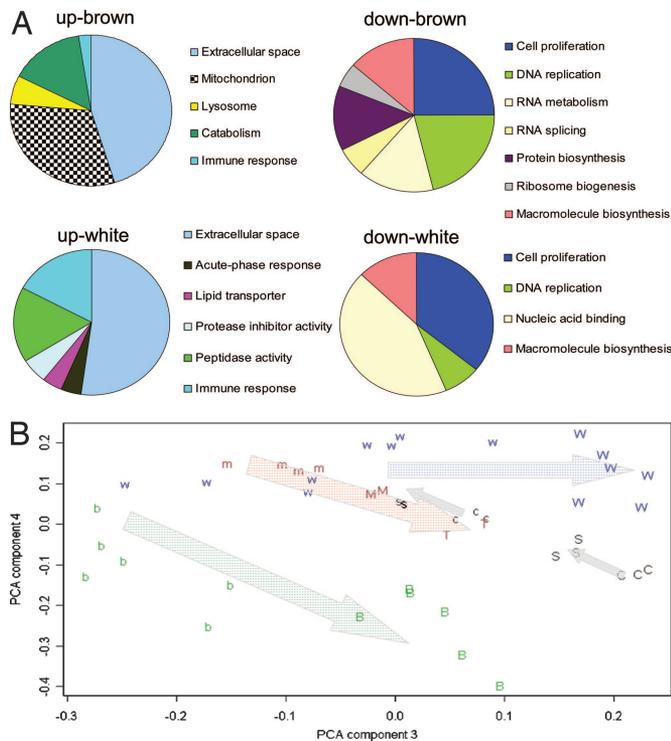


Fig. 3. GO analysis of differentiating brown and white primary preadipocytes compared with C2C12 myoblasts and PCA. (A) Diagrammatic representation of the GO analysis carried out by using EASE. GO subgroups were collapsed into related groups to facilitate visualization of the data. For the complete breakdown of significantly enriched gene ontology groups, see [SI Data Set 3](#). All GO groups demonstrated enhanced statistical representation (based on an EASE score and a Bonferroni-adjusted value of $P < 0.05$). (B) We used a PCA-based approach to compare the C2C12 differentiation data set, the SIRT1 differentiation data set (where SIRT1 overexpression prevents myoblast differentiation) with the differentiating pre-brown adipocytes and pre-white adipocytes. The data set includes day 4 undifferentiated brown (b) and white (w) preadipocytes and day 7/8 differentiating brown (B) and white (W) preadipocytes. C2C12 myoblasts are shown as m, and progressively differentiating myoblasts are plotted as M and T (<http://pepr.cnmcresearch.org/>). Sirt1-overexpressing cells (16) (s for 12-h differentiation and time control c, whereas S is 36-h differentiation with SIRT1 viral overexpression and time control C). After global normalization, we examined the top 100 genes that contributed to components 3 to 5 and used GO analysis to identify the putative SIRT1-regulated genes (see *Methods*). Components 3 and 4 are plotted. The arrows are used to illustrate the general direction of cell differentiation. It should be noted that SIRT1 (s and S) overexpression opposes the differentiation process.

establish a large common transcriptional profile even between primary brown and white preadipocytes, the lack of commonality between the *IRS1*^{-/-} adipocyte cell line and brown preadipocytes is perplexing but apparently robust.

SIRT1 as a Plausible Brown Adipocyte Differentiation Regulator. Mitochondrial abundance is important to both brown adipocyte (5) and skeletal muscle differentiation and function (31, 32). The Sir2 ortholog SIRT1 would represent an excellent candidate for carrying out the dual role of suppressing the myogenic signature because it prevents myoblast differentiation (16) as well as promoting mitochondrial biogenesis as it can modulate PGC1 α action through its class III histone deacetylase activity (33). The role of SIRT1 in brown adipocytes has not been studied, and thus we used the transcriptome changes generated after SIRT1 overexpression in myoblasts (16) to evaluate the possible role of SIRT1 during brown preadipocyte differentiation. A total of 40 genes significantly overlapped between SIRT1 down-regulated genes and genes down-

regulated during brown preadipocyte maturation ([SI Data Set 5](#)). This overlapping gene list was very specific, being highly enriched with muscle development genes ($P < 0.00003$ after Bonferroni correction) and thus was clearly nonrandom. Further, we found that 59 genes were common to SIRT1 up-regulated genes and genes up-regulated during brown preadipocyte maturation; this list was highly enriched for extracellular matrix genes and antigen-responsive genes ($P < 0.0003$ after Bonferroni correction).

The potential importance of the SIRT1-related overlapping gene lists was also assessed by using an approach to PCA (20, 21), where we combined PCA with GO analysis to give the top 100 ranking genes within a component some biological context. During differentiation of all cell types studied (or inhibition of differentiation in the case of SIRT1 overexpression in myoblasts), the first few components reflected modulation of ribosomal, cell cycle, and RNA regulation genes (e.g., Fig. 3B, component 2, and [SI Data Set 6](#), component 3). Compellingly, component 4 (Fig. 3B) captured a variance category that described the maturation of brown preadipocyte and C2C12 muscle cells and the negative impact of SIRT1 overexpression (16) on differentiation, whereas this component was neutral for white adipocyte differentiation ([SI Data Set 6](#)). Consistent with our hypothesized role for SIRT1 in brown preadipocyte differentiation, component 4 represented mitochondrial and metabolism genes ($P < 0.00001$ after Bonferroni correction; [SI Data Set 6](#)). Component 5 ([SI Data Set 6](#)) was statistically enriched for muscle development genes where both SIRT1 overexpression and brown preadipocyte differentiation are associated with the down-regulation of this common group of muscle-specific genes, also supporting our hypothesis. These substantial and biologically distinct overlapping transcriptional signatures were in stark contrast to the lack of similarity between the primary brown adipocytes and the *IRS1*^{-/-} brown adipocytes, suggesting that SIRT1 would be a valid and interesting target for further biochemical studies.

TF Analysis. Although our analysis has provided a unique insight into primary preadipocyte maturation, identification of the TFs potentially responsible for these coordinated gene expression programs would further enhance our understanding. To address this aim, we carried out *in silico* prediction analysis ([SI Fig. 6](#)) of TF-binding sites within the upstream regions of all genes on the Affymetrix U74A v2 array and then compared them with the frequency of occurrence within each of the modulated gene lists from brown and white preadipocytes during differentiation ([SI Data Set 2](#)). Robust prediction of TF sites in eukaryotic genes is challenging because of the degenerative nature of binding sites (34) that yields a high rate of false-positive predictions. We therefore used phylogenetic footprinting (35), using human sequences for comparison, and restricted our analysis to only the 1,000 bp upstream. ClustalW (36) alignments between upstream mouse and corresponding human sequences were scanned for TF-binding sites by using the TFBS software (37) and JASPAR database (38). Between 12 and 39% of all TF-binding sites within the JASPAR database (111 sites) were significantly underrepresented within each modulated gene list. Only one TF-binding site, that for E2F, was overrepresented ([SI Data Set 7](#)), and it was in the list of genes down-regulated during brown adipocyte differentiation. It is unclear why the modulated gene lists were specifically underenriched for TF-binding sites, but the general outcome most likely indicates that the repertoire of the JASPAR database is still limited.

Discussion

Although both adipocytes and myocytes can differentiate from a common mesenchymal precursor cell (39), our analysis indicates that brown adipocytes endogenously share a common early transcriptional program with skeletal muscle cells, which is then suppressed early during differentiation. There was no Tcf21 expression in brown adipocytes, which would suggest that Tcf21 may be the first useful positive selection marker for white

CA)/2.4 nM insulin/25 μ g/ml sodium ascorbate/10 mM HEPES, pH 7.4/4 mM glutamine/50 units/ml penicillin/50 μ g/ml streptomycin. To verify the cell phenotype (using *ucp1* expression as a marker), cells were treated with either vehicle or 0.1 μ M norepinephrine for 4 h, all as described previously (5).

Microarray Analysis and RT-qPCR and Northern Blot Analysis. We used the Affymetrix (Santa Clara, CA) U74A v2 array platform, and all array data were normalized through implementations of the MAS5 algorithm and then subjected to a multilevel gene array analysis strategy. Full details can be found in *SI Methods*.

For RT-qPCR, total RNA was isolated by the use of Ultraspec (Biotech, Houston, TX) followed by DNase treatment with the RNeasy mini kit column procedure (Qiagen, Valencia, CA). RNA (750 ng) was reverse-transcribed by reverse transcription reagents (Applied Biosystems, Foster City, CA) with random hexamer primers, in a total volume of 40 μ l. RT-qPCR aliquots contained 2–5 μ l of the sample cDNA, 2 \times TaqMan universal PCR master mix, and an optimized concentration of each primer, added to a final volume of 24–26 μ l and were measured in triplicate from three independent cell cultures. For the muscle-specific factors (MyoD, myogenin, Myf5 and Myf6), hindlimb skeletal muscle was obtained from 4-week-old animals to act as a positive control. In this situation, expression was normalized first to 18S and then to expression levels found in skeletal muscle. For all other genes, data were normalized to 18S and then

expressed as a percentage of the sample with the highest expression (shown as 100% in Fig. 2). Northern blotting was carried out as described in *SI Methods*.

Immunohistochemistry. Primary muscle cells were plated in 6-well plates containing collagen-coated coverslips. The cells were rinsed in PBS and before fixing in 4% paraformaldehyde for 20 min at room temperature. Fixed cells were washed in PBS containing 0.2% fish skin gelatin, permeabilized in 0.2% Triton X-100 for 4 min, and then blocked in goat serum for 10 min. The coverslips were then incubated in primary antibody (1:50) for 20 min at room temperature and washed in 0.2% fish skin gelatin. Primary antibody binding was detected after incubation with Cy3-labeled secondary antibodies (1:500) and mounting in Vectashield (Vector Laboratories, Burlingame, CA). Adipocytes were grown on coverslips in 6-well plates and were washed in 37°C PBS and fixed in 3.7% formaldehyde in PBS, then permeabilized in 0.1% Triton X-100 in PBS for 2 min at room temperature and processed as above.

We thank Birgitta Leksell for technical assistance. This work was supported by Swedish Science Research Council Grant 621-2004-3858 (to B.C. and J.N.), Swedish Cancer Foundation Grant 05-0291 (to J.N.), Swedish Diabetes Society Grant DIA2005-004 (to J.T.), VINNOVA Grant P25770-1 (to C.W.), and funding from Heriot-Watt University Grant L6004 (to J.T.).

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