Genetic variability affects the development of brown adipocytes in white fat but not in interscapular brown fat

Bingzhong Xue, Jong-Seop Rim, Jessica C. Hogan, Ann A. Coulter, Robert A. Koza, and Leslie P. Kozak

Pennington Biomedical Research Center, Baton Rouge, LA 70808

Abstract Cold exposure induces brown adipocytes in retroperitoneal fat (RP) of adult A/J mice but not in C57BL/6J (B6) mice. In contrast, induction of the mitochondrial uncoupling protein 1 gene (Ucp1) in interscapular brown adipose tissue (iBAT) shows no strain dependence. We now show that unlike iBAT, in which Ucp1 was expressed in the fetus and continued throughout life, in RP, Ucp1 was transiently expressed between 10 and 30 days of age and then disappeared. Similar to the lack of genetic variation in the expression of Ucp1 in iBAT during cold induction of adult mice, no genetic variation in Ucp1 expression in iBAT was detected during development. In contrast, UCP1-positive multilocular adipocytes, together with corresponding increases in Ucp1 expression, appeared in RP at 10 days of age in A/J and B6 mice, but with much higher expression in A/J mice. At 20 days of age, brown adipocytes represent the major adipocyte present in RP of A/J mice. The disappearance of brown adipocytes by 30 days of age suggested that tissue remodeling occurred in RP. Genetic variability in Ucp1 expression could not be explained by variation in the expression of selective transcription factors and signaling molecules of adipogenesis. In summary, the existence of genetic variability between A/J and B6 mice during the development of brown adipocyte expression in RP, but not in iBAT, suggests that developmental mechanisms for the brown adipocyte differentiation program are different in these adipose tissues.—Xue, B., J-S. Rim, J. C. Hogan, A. A. Coulter, R. A. Koza, and L. P. Kozak. Genetic variability affects the development of brown adipocytes in white fat but not in interscapular brown fat. J. Lipid Res. 2007. 48: 41–51.

Supplementary key words white fat development • mitochondrial uncoupling protein 1 • transcription factors of adipogenesis • immunoblot analysis • quantitative reverse transcription polymerase chain reaction • immunohistology • peroxisome proliferator-activated receptor α knockout • peroxisome proliferator-activated receptor γ coactivator-1α • A/J mice • C57BL/6J mice

Since the cloning of leptin, we have come to appreciate that the adipocyte is not just a place for storing energy as fat but is also an endocrine cell, secreting cytokines associated with the control of energy balance (1, 2). Adipose tissue has been shown to have additional potentialities at the cellular level, as shown by the presence of mesenchymal stem cells in the tissues that are able to differentiate into several different cell types (3). However, a potentiality of particular interest to obesity research is the striking cellular transformation that occurs when a fat depot composed almost exclusively of white adipocytes, in which fat is stored, is converted to a site enriched in brown adipocytes, in which fat is oxidized to produce heat (4, 5). Thus, two diametrically opposed functions can be performed by the same adipose tissue, depending on the cellular environment and genetic constitution of the animal.

Heat generated through the uncoupling of oxidative phosphorylation via uncoupling protein 1 (UCP1) in brown adipose tissue maintains body temperature in small and newborn mammals exposed to cold (6, 7). Consistent with this function, the defined interscapular brown adipose tissue (iBAT) depots develop precociously during fetal development to provide the newborn with protection from cold exposure (8). Brown adipocytes, although not present to any extent in traditional white fat depots of adult rodents maintained at 25°C, can be induced in these depots in adult animals (9, 10). Because the development of white fat depots occurs almost exclusively during the postnatal period immediately after birth (11), the developmental dynamics during this period that provide white fat depots with the potentiality to activate a brown adipocyte differentiation program in the adult animal represent an intriguing area for investigation. Several lines of evidence suggest that a
function for brown adipocytes in white fat depots lies in the
regulation of energy balance and body weight through
enhanced thermogenesis. Since the initial hypothesis on
diet-induced thermogenesis by Rothwell and Stock (12) in
1979, many pharmacological and genetic studies in animals
have shown correlations between increased UCP1 in white
fat depots and reduced obesity. Ghorbani and Himms-Hagen (13)
showed that the reversal of obesity and diabetes in Zucker fa/fa
rats by the β3-adrenergic agonist CL 316,243 is accompanied by the appearance of brown adipocytes in
white fat. Studies in inbred strains A/J and C57BL/6j (B6)
also revealed that the ability of CL 316,243 treatment to
prevent diet-induced obesity in A/J mice correlated with the
induction of brown adipocytes in white fat regions (5, 14).
In addition, several transgenic studies have demonstrated that increasing
UCP1 expression in white fat is associated with the reduction of adiposity (15–19).
The induction of Ucp1 expression in iBAT of adult A/J
mice is quantitatively indistinguishable from that of B6
mice (20). In contrast, A/J mice show a strong induction
of brown adipocytes in retroperitoneal fat (RP) depots by
adrenergic stimulation, whereas B6 mice are largely re-
sistant to this induction (5). These patterns of expression
suggest that if differences in nonshivering thermogenesis
provide the mechanistic bases for susceptibility to obesity
and insulin resistance between the two strains, then it is
the brown adipocytes in white fat depots that are respon-
sible for the phenotypic variation in obesity, and not iBAT.
Based upon this rationale, our laboratory pursued a quan-
titative trait locus (QTL) analysis of Ucp1 induction in the
RP (5, 21, 22). As many as eight individual QTLs that
control Ucp1 mRNA levels in RP after cold exposure for
7 days were mapped to the mouse genome. With the
exception of the peroxisome proliferator-activated receptor
(PPAR) α gene on chromosome 15, candidates for the
other QTLs have not been identified. However, several
components in both signaling and transcription, including
p38 mitogen-activated protein kinase, cAMP response
element binding protein (CREB), type 2 T4 deiodinase
(Dio2), peroxisome proliferator-activated receptor γ co-
activator-1α (Pgc1α), and Pparα, were shown to be only
modestly, but significantly, upregulated by these QTLs.
Therefore, it was proposed that these regulatory factors
interact synergistically to effect a large 80-fold difference
in expression of Ucp1 between A/J and B6 mice (22).
It is intriguing that genetic variation is observed for
brown adipocyte induction in the RP, whereas there is no
variation in the induction of Ucp1 in iBAT of adult mice
during cold exposure. One explanation is that the genetic
differences in the induction of the brown adipocytes in RP
of A/J and B6 adult mice reflect the recapitulation of dif-
fferences in the mechanisms controlling the induction of
the brown adipocyte phenotype that was established dur-
ing early postnatal development, whereas induction of
Ucp1 in iBAT is simply a physiological response of mature
differentiated brown adipocytes. A second related ques-
tion concerns the origin of those adipocytes in RP that
become differentiated brown adipocytes. There is mor-
phological evidence that during the induction of brown
adipocytes in adult mice, cell division is essentially un-
detectable (23) and the induction of Ucp1 reaches max-
imal levels within 2 days, a kinetic response that is more
consistent with the conversion of mature white adipocytes
into brown adipocytes (5) than with de novo brown adi-
pocyte differentiation from preadipocytes.

To address these questions, it is necessary to have basic
information on the molecular features of the develop-
mental program for adipogenesis in the RP depot in A/J
and B6 mice that is comparable to what we now have for
the RP in adult mice during cold exposure (5, 22). This
developmental analysis has revealed that an unexpected
cellular remodeling of a white fat depot occurs during
postnatal development, in which a majority of the adi-
pocytes in the tissue between 10 and 30 days of age are
multilocular and express UCP1, followed by the loss of
the multilocular brown adipocytes and the reemergence
of unilocular white adipocytes. This leads to the formation
of traditional white fat in adult animals that appears to
be composed exclusively of white adipocytes and devoid
of brown adipocytes; subsequently, adrenergic stimula-
tion of the adult mouse can lead to the reappearance
of brown adipocytes.

MATERIALS AND METHODS

Animals

Breeding pairs of the A/J, B6, and 129Sv/ImJ strains and the
Pparα knockout mice were obtained from the Jackson Laboratory
(Barr Harbor, ME) and bred at the Pennington Biomedical Re-
search Center. All parental mice and their pups were kept at
room temperature (23 ± 1°C) with a 12 h light/dark cycle.
Mice were ad libitum fed a chow diet (11.9 kcal% fat and
64.5 kcal% carbohydrate; Picolab 5053 Rodent Diet 20),
and pups were weaned at 21 days of age onto the same chow diet.
To study brown adipose tissue development during the perinatal
stage, male and female A/J or B6 mice were mated, and the
day of conception was determined by the presence of a vaginal
plug. iBAT was collected from fetuses after 18–20 days of ges-
tation, newborn pups (0 h), and 24 h old pups (24). Male mice
with ages ranging from 5 days to 4 months were euthanized by
cervical dislocation, and iBAT and RP were removed for vari-
ous measurements.

Real-time RT-PCR

Total RNA was isolated from tissues using Tri-Reagent (Mo-
lecular Research Center, Cincinnati, OH). RNA expression levels
for Ucp1 and other genes were measured by quantitative real-time
RT-PCR using the ABI Prism 7700 and 7900 sequence detection
systems (Applied Biosystems, Foster City, CA), as described pre-
niously (21). Briefly, RNA was diluted to 50 ng/μl (RP) or 20 ng/μl
(iBAT) in formamide and then diluted 1:100 in deionized water
just before use. Ten microliters of diluted RNA (5 ng for RP and
2 ng for iBAT) was used in a 50 μl reaction with a single reporter
measurement. The sequences for the primers and probes are
available upon request. RNA from liver was used as a standard for
PPARα, and pooled iBAT RNA from 18–20 day fetus was used as a
standard for Preadipocyte factor 1. Standards for the remaining
genomes were prepared as described previously (21). Both standards
and samples were run in duplicate. Each transcript was corrected
by the level of cyclophilin mRNA.
Mitochondrial DNA measurement

Total DNA was isolated using Tri-Reagent after the isolation of RNA according to the manufacturer’s instructions. Mitochondrial mass was determined by measuring mitochondrial DNA-encoded cytochrome c oxidase subunit I (COXI) by real-time PCR using a Taqman probe and primers for COXI. COXI levels were normalized to UCP2, a nuclear DNA-encoded gene DNA. IBAT DNA was used as a standard.

Western blot analysis

Analysis of iBAT and RP tissues was carried out as described by Xue et al. (22). Rabbit anti-UCP1 (1:10,000) and anti-PGC1α (1:4,000) polyclonal antibodies were kindly provided by Dr. Thomas Gettys (25). Rabbit anti-PPARα (1:1,000) and anti-PPARγ (1:1,000) polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies against protein kinase A (PKA) subunits (Ca, RIα, RIIα, pRIIβ, and RIIβ) were obtained from BD Transduction Laboratories (San Jose, CA). Rabbit antibodies against CREB, Activating Transcription Factor (ATF), and mitogen-activated protein kinase were obtained from Cell Signaling Technology (Beverly, MA).

Immunohistochemistry

RP tissues were fixed in 4% paraformaldehyde, paraffin-embedded, cut into 3 μm thick sections, and mounted onto SuperFrost Microscopy Slides (Fisher Scientific, Pittsburgh, PA). Immunohistochemistry for UCP1 was performed according to Fekete et al. (26) with some modifications (5).

Dio2 activity measurement

Dio2 activity was measured with 2 nM $^{125}$I-T4 (specific activity, 1,000 μCi/μg; Perkin-Elmer, Boston, MA) purified using LH-20 column chromatography (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) on the day of the experiment, according to Schneider et al. (27). Nonenzymatic deiodination was corrected for by subtracting I$^2$ released in tissue-free measurements. Dio2 activity was reported as fmol I$^2$ released/mg/h. Results were multiplied by 2 to correct for random labeling at the equivalent 3’ and 5’ positions.

RESULTS

Body weight and fat pad mass during development

No strain differences were observed in body weights and iBAT or RP mass between A/J and B6 mice from late gestational development through the postnatal period and until mice were 2 months of age (Fig. 1). Slight differences were found at 4 months of age. In addition, there was no difference in litter size between the two strains (7 ± 2 for A/J and 7 ± 1 for B6), which excludes the possible influence of litter size on sympathetic nervous system development of the pups (28).

Induction of UCP1 expression in iBAT during development

$Ucp1$ mRNA and protein levels were first detected in fetal iBAT in both A/J and B6 mice after 18 days of gestation and showed an equally rapid increase toward birth (Fig. 2A, B). UCP1 mRNA reached maximal levels at 1 day of age, and protein levels reached peak values at 10 days after birth and then both remained constant, although a slight decrease was observed in B6 mice. In addition, there was a parallel increase in mitochondrial content in iBAT during this period, as measured by the amount of COXI DNA, a mitochondrial DNA-encoded gene (Fig. 2C). These patterns of brown adipocyte expression are consistent with those observed by others (24, 29–32) and with previously published results from our laboratory with B6 mice (33). No strain-dependent variations in either $Ucp1$ expression or mitochondrial amount in iBAT were detected between A/J and B6 mice from 18 days of gestation to 4 months of age.

Induction of brown adipocytes in RP during development

We next investigated the appearance of brown adipocytes in white fat during early postnatal development. $Ucp1$ mRNA and protein levels in RP of both A/J and B6 mice were very low at 5 days of age, although they were 10-fold higher in A/J mice ($Ucp1$ mRNA and protein levels in RP of both A/J and B6 mice were very low at 5 days of age, although they were 10-fold higher in A/J mice. $Ucp1$ mRNA levels increased in both strains, reaching a maximum for B6 mice at 10 days of age and for A/J mice at 20 days of age, after which it remained
increased until 30 days of age before declining (Fig. 3A). The expression of UCP1 protein generally agreed with the mRNA data, although the level of protein, measured in a separate set of mice, began its decline after 20 days of age (Fig. 3B). Although increases in Ucp1 expression initiated at approximately the same time for both strains, the induction in B6 seemed to abort early, somewhere between 10 and 20 days of age, and declined thereafter. On the other hand, expression in A/J mice increased until 20 days of age, resulting in a 5-fold higher level of expression than in B6 mice; however, expression eventually decreased in the A/J mice as well (Fig. 3A, B). Mitochondrial DNA in RP also showed a parallel increase during the early postnatal stage, reached peak levels at 1 month of age, and decreased slightly after that (Fig. 3C). The level of mitochondrial DNA was higher in A/J mice than in B6 mice (Fig. 3C).

The Ucp1 expression data and the analysis of COXI DNA for mitochondrial content indicating a higher brown adipocyte content in A/J mice were confirmed by UCP1 immunohistochemistry. A field of cells dominated by those with the morphology of brown adipocytes indicates that brown adipocytes transiently became the major adipocyte of RP between ~20 and 30 days of age (Figs. 4, 5). A mon-

![Fig. 2. Development of iBAT in A/J and B6 mice. Uncoupling protein 1 (Ucp1) mRNA (A), UCP1 protein (B), and mitochondrial DNA (C) expression in iBAT of A/J and B6 mice during prenatal and postnatal development. For Ucp1 mRNA, each time point is the average value from four to nine individual animals analyzed in duplicate and is expressed relative to cyclophilin. Cytochrome c oxidase subunit I (CoxI) mitochondrial DNA is expressed relative to the nuclear gene Ucp2. Four mice were analyzed in duplicate. AU, arbitrary units.](image1)

![Fig. 3. Induction of Ucp1 in RP of A/J and B6 mice during postnatal development. Ucp1 mRNA (A), UCP1 protein (B), and mitochondrial DNA (C) expression. * P < 0.05 versus B6 mice. Ucp1 mRNA data are based upon the analysis of three to six animals, and CoxI DNA data are based upon the analysis of four mice per time point. AU, arbitrary units.](image2)

![Fig. 4. Immunohistochemical assessment of the appearance of UCP1-positive multilocular brown adipocytes in RP of A/J and B6 mice during postnatal development. Magnification, ×20.](image3)
tage of microscopic fields from A/J and B6 mice shows that the majority of cells in the RP are multilocular (Fig. 5). In RP of A/J mice, these cells are clearly positive for UCP1 by immunohistology; however, in B6 mice, it is difficult to discern staining for UCP1 because of the low UCP1 expression. Low-power micrographs of RP from four mice show that the majority of adipocytes in the RP of 20 day old mice were brown adipocytes (see supplementary Fig. I). Brown adipocyte induction in RP of adult mice is mosaic, with some strains (AXB8) showing an abundance of regions with brown adipocytes and others (AXB15) having few brown adipocytes (5) (see supplementary Fig. II). Accordingly, the dominant appearance of brown adipocytes in the RP of 20 day old mice was surprising. The acquisition of brown adipocytes in B6 mice occurred more transiently, as there were no multilocular brown adipocytes present in RP by the time they were 1 month old, whereas an abundance of brown adipocytes still existed in RP of A/J mice at the same age. However, by the time A/J mice were 2 months of age, all brown adipocytes disappeared and were replaced by unilocular white adipocytes (Fig. 4). Cold exposure of 2 month old animals for 7 days resulted in the induction of brown adipocytes in RP of A/J mice but not B6 mice (5, 22). Immunohistology of white adipose tissue in adult tissues of mice shows a broad range of multilocular cells with UCP1, but rarely if ever do multilocular cells not express UCP1. Immunohistology of iBAT with an antibody that is highly specific for UCP1, as shown by the absence of staining with control serum and in tissue from Ucp1-deficient mice (see supplementary Fig. III), also shows variation in the intensity of staining, but multilocular cells have protein that almost always reacts with the antibody.

To provide a comparison of Ucp1 expression in iBAT and RP in two strains of mice and at two stages of devel-

Fig. 5. A montage of photomicrographs of A/J and B6 RP immunohistograms stained with antibody against UCP1 and counterstained with hematoxylin. More than 80% of the RP depot is present in the figure. Magnification, 20×. (See supplementary data for patterns of staining in additional mice.)
expression, its expression at the mRNA level, as determined by quantitative RT-PCR, is presented in Table 1. In iBAT, Ucp1 expression in A/J and B6 mice is similar at 20 and 60 days of age at 23°C, and it is comparably induced in A/J and B6 mice by cold at 60 days of age. In contrast, Ucp1 expression in RP of A/J mice is always higher than in B6 mice, and induction by cold exposure is very low in the RP of B6 mice but very strong in A/J mice, in which levels reach 71% of those observed in iBAT at ambient temperature (25°C). We do not know the levels of Ucp1 expression that could be induced in RP by cold exposure of 20 day old mice.

Expression of transcription factors in RP during development

The genetic analysis of Ucp1 induction in RP has led to a model for regulation that occurs on two levels (22). On the first level, allelic differences in eight QTLs determine variation in selective signaling and transcription factors in RP of A/J and B6 mice. Because the magnitude of the increase in these factors did not approach that observed for cold induction of Ucp1, we proposed a second level of regulation in which modest differences in the transcription factors and signaling molecules achieve a much higher effect in transcribing a target gene if they interacted synergistically in the regulation of Ucp1. Given that variation in Ucp1 expression between A/J and B6 mice also exists in developing RP, if the same mechanisms regulating gene expression in the adult mice during cold-induced gene expression were also operating in the developing RP depots, then variation in these factors might also exist there. Accordingly, we have determined the profiles of these molecules in the developing RP of A/J and B6 mice.

The developmental profiles of several transcription factors showed the expected increase in expression during postnatal RP development, consistent with roles for these factors in adipogenesis, as established previously from studies with the 3T3-L1 cell culture model. A peak in the expression at ~20 days of age was observed for Pparg, sterol regulatory element binding protein 1c, Cebp, C, and β, adipocyte P2 (aP2), Pgc1α, and Pparβ; although none of them returned to the baseline as Ucp1 did (Figs. 6, 7). This is consistent with the brown adipocyte specificity of Ucp1, as the other genes are also expressed in white adipocytes and possibly other cells in the developing fat tissue. Pre-adipocyte factor 1, a putative marker for the preadipocyte, decreased to very low levels by 20 days of age after high expression at 5 days of age in RP tissue from A/J and B6 mice (Fig. 6G).

Besides Coxl DNA (Fig. 3C), only Pgc1α and Pparβ showed significant increases in expression at both mRNA and protein levels in the RP from A/J mice compared with B6 mice (Fig. 7). The 4-fold induction of Pparβ, comparable to the magnitude of Ucp1 induction, suggested a significant role for PPARβ in the developmental regulation of RP remodeling. However, the induction of Ucp1 in Pparβ knockout mice on the 129Sv/ImJ background at 20 days of age was not significantly different from that in wild-type controls (Fig. 7E). 129Sv/ImJ mice have increased Ucp1 expression during cold acclimation, similar to the A/J strain. With the exception of Ucp1 expression, Dio2 expression showed the most robust differences between A/J and B6 mice during the cold adaptation of adult mice (22); however, no differences could be detected in the RP during postnatal development, and it is surprising that there was not even a spike of activity at 20 days of age when the expression of Ucp1 was maximal (Fig. 8).

PKA-associated signaling

We previously showed that during cold induction of Ucp1 in RP of 2 month old mice, subunits of PKA showed no change in expression between A/J and B6 mice by immunoblot analysis. Yet, modest 3- to 5-fold increases in phospho-CREB and phosphorylated p38 mitogen-activated protein kinase were detected in A/J mice after 1 and 7 days of cold exposure compared with B6 mice (22). A similar analysis of RP from A/J and B6 mice during postnatal development was performed. Figure 9A confirms the data in Fig. 3B showing that the levels of UCPI were highly increased in A/J mice compared with B6 mice. In this set of tissues, differences in PGC1α protein levels were small. Analyses of subunits of PKA, CREB, ATF, p38 mitogen-activated protein kinase, extracellular regulated kinase, and stress-activated protein kinase/c-Jun kinase do not indicate that these signaling

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Age/Conditions</th>
<th>Ucp1</th>
<th>Relative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A/J</td>
<td>B6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arbitrary units/cyclophilin mRNA</td>
<td>A/J</td>
</tr>
<tr>
<td>iBAT</td>
<td>20 days/23°C</td>
<td>773 ± 31 (n = 11)</td>
<td>789 ± 9 (n = 7)</td>
</tr>
<tr>
<td></td>
<td>60 days/23°C</td>
<td>998 ± 27 (n = 3)</td>
<td>598 ± 58 (n = 6)</td>
</tr>
<tr>
<td></td>
<td>60 days/4°C, 1 day</td>
<td>1,766 ± 27 (n = 4)</td>
<td>1,352 ± 28 (n = 3)</td>
</tr>
<tr>
<td></td>
<td>60 days/4°C, 7 days</td>
<td>1,847 ± 73 (n = 5)</td>
<td>1,834 ± 70 (n = 3)</td>
</tr>
<tr>
<td>RP</td>
<td>20 days/23°C</td>
<td>63 ± 14 (n = 5)</td>
<td>6 ± 1.4 (n = 4)</td>
</tr>
<tr>
<td></td>
<td>60 days/23°C</td>
<td>7 ± 1.4 (n = 5)</td>
<td>0.2 ± 0.04 (n = 6)</td>
</tr>
<tr>
<td></td>
<td>60 days/4°C, 1 day</td>
<td>234 ± 63 (n = 5)</td>
<td>3 ± 1.6 (n = 3)</td>
</tr>
<tr>
<td></td>
<td>60 days/4°C, 7 days</td>
<td>547 ± 59 (n = 5)</td>
<td>6 ± 1.0 (n = 3)</td>
</tr>
</tbody>
</table>

B6, C57BL/6j; iBAT, interscapular brown adipose tissue; RP, retroperitoneal fat; Ucp1, uncoupling protein 1 gene.

TABLE 1. Ucp1 mRNA levels in iBAT and RP in A/J and B6 mice at 20 and 60 days of age and in mice maintained at 23°C or 4°C
pathways have been upregulated in A/J mice in a manner that could account for higher levels of \textit{Ucp1} transcription.

**DISCUSSION**

This investigation comparing the development of iBAT and RP in two inbred strains of mice was initiated to understand the origins of brown adipocytes that are induced in white fat depots. We reasoned that understanding why genetic variability is associated with the induction of brown adipocyte phenotypes in traditional white fat depots such as RP, but not in a defined brown adipocyte depot such as iBAT, may provide critical insights. We hypothesized that the manifestation of genetic variability was a function of developmental age. Brown adipocytes are already present and differentiated in the iBAT of adult mice, so adrenergic stimulation acts on cells with fully developed molecular mechanisms to induce \textit{Ucp1}. This physiologically associated gene induction in fully differentiated brown adipocytes is not subject to genetic variability. On the other hand, a major differentiation process occurs in adult RP upon adrenergic stimulation, as shown by the morphological conversion of white to brown adipocytes (5). Accordingly, the early development of iBAT in the fetus was hypothesized to be subject to genetic variability. The data from this study clearly rejected this hypothesis: there was no evidence for variability in \textit{Ucp1} expression in iBAT between A/J and B6 mice from 17 days of gestation up to 4 months of age.

In contrast to the similarity of iBAT development in A/J and B6 mice, a strikingly different phenotype was observed in the developing RP depots of these strains. The developmental profiles for UCP1 and brown adipocytes in the RP were very different for A/J and B6 mice and resembled those previously shown to occur during cold-induced brown fat induction in RP of adult mice (5, 22). The data suggest that this variable strain response to cold exposure reflects a recapitulation of brown fat adipogenesis in white fat depots of adult mice that had previously occurred at an ambient temperature of 23°C in postnatal white fat depots during lactation. The genetic variability can be con-
sidered to be a signature of brown adipogenesis in white fat depots, whether it occurs during postnatal development or in adrenergically stimulated adult mice. Because iBAT does not possess this phenotype, a fundamental difference seems to exist between the developmental mechanisms controlling brown adipogenesis in iBAT and RP.

This appearance of brown adipocytes in traditional white fat depots of mice during postnatal development was surprising, because it had not been described previously in rodents (34, 35). While this study was in progress, two reports appeared describing the transient appearance of brown adipocytes in developing mammary gland at approximately the same stage of postnatal development as we found (36, 37). The functions of these brown adipocytes in mammary glands were proposed in one study to contribute to adaptive thermogenesis (38) and in the other to the regulation of the mammary epithelium (36). Additionally, there is evidence for multilocular adipocytes in subcutaneous fat of 1–2 month old mice (38), consistent with the higher levels of Ucp1 expression in subcutaneous fat depots compared with those in the peritoneum (5). Although the transient appearance of brown adipocytes in white fat depots during development may be novel for the rodent, it has been known to be a basic feature of early adipogenesis in cattle, sheep, and other large animals that are born in early spring and therefore have a need for thermogenesis to survive (39–42). The limited human data can also be interpreted to indicate that a similar pattern of adipocyte development and differentiation occurs in white fat depots for survival in the cold upon birthing (43). Accordingly, the transient appearance of brown adipocytes in white adipose tissues during early development may be a basic feature of mammalian species independent of the size of the animal. Those discrete brown fat depots located in the interscapular regions and around vital organs such as the heart and kidneys that persist in a highly differentiated state throughout life should be considered as providing an additional layer of thermogenesis to protect vital organs in animals with a large surface-to-volume ratio.

To understand the striking difference in Ucp1 expression and brown adipocyte differentiation in the RP of A/J and B6 mice, we analyzed the transcription factors and signaling molecules for adipogenesis that have been delineated by in vitro studies in 3T3-L1 cells. The expression pattern of these genes in RP during the first month after birth recapitulated the in vitro differentiation process observed in 3T3-L1 fibroblasts, suggesting that the first postnatal month is critical for white adipose tissue development and maturation in vivo; however, the expression of none of these transcription factors was variable between A/J and B6 mice. This is predictable, because these factors are involved in both white and brown adipogenesis, both of which occur in developing RP. More pertinent to explanations for the difference in Ucp1 expression are the

![Image of graphs and figures](image-url)
variations in PGC1α and PPARα. These two factors, which have been implicated specifically in brown adipogenesis, were expressed with a pattern that resembled Ucp1 expression, and they were variable between A/J and B6 mice. This suggested that PPARα may be necessary for the variable expression and that its expression may be rate limiting for brown adipogenesis. We tested this idea by analyzing the effect of the PPARα knockout allele on Ucp1 expression (44), but there was no reduction in Ucp1 expression in the knockout mice. Although this result is difficult to understand, given the evidence that PPARα is involved in Ucp1 expression, the fact that the PPARα knockout mice and the wild-type controls were on the 129SV/ImJ genetic background may underscore the importance of genetic makeup on the expression of variable phenotypes for Ucp1. Furthermore, identical results were obtained when the induction of Ucp1 in RP by cold exposure was compared in adult PPARα knockout and wild-type controls. This provides additional genetic evidence for the developmental equivalence of brown adipocytes in the RP of 10–20 day old mice and 2 month old mice. A caveat to the equivalence of brown adipocytes in white fat from neonatal and adult mice is found in Dio2 regulation, as a robust differential induction of Dio2 in RP occurs in adult mice during cold adaptation between A/J and B6 animals (22), whereas there is no strain difference in Dio2 expression and activity during postnatal development (Fig. 8). Therefore, differences in the genetic control of Ucp1 induction by thyroid hormone-associated pathways exist between postnatal development and cold adaptation in adult mice.

The characteristics of brown adipocyte development offer an opportunity to explore the interplay of both ontogenetic and environmental factors in the establishment of a complex tissue. In a model of this development (Fig. 10), brown adipocytes first appear in a rapid burst of expression in the interscapular fat during the last 3 days of gestation. Experiments demonstrating that Ucp1 mRNA levels do not increase in iBAT of newborn pups when the ambient temperature has been maintained at thermoneu-

Fig. 8. Type 2 T4 deiodinase (Dio2) mRNA (A) and Dio2 enzyme (B) activity in RP of A/J and B6 mice during postnatal development. Both Dio2 mRNA and enzyme activity are based upon the analysis of three mice for each time point. A.U., arbitrary units.

Fig. 9. Protein expression and phosphorylation patterns for UCP1 and cell signaling components in total tissue extracts of RP from A/J and B6 mice during postnatal development. A: UCP1 and PGC1α expression. B: Catalytic and regulatory subunits of protein kinase A. C: CREB and activating transcription factor (ATF). D: Mitogen-activated protein kinases. 20d, 27d, and 60d refer to postnatal age in days. Each protein sample is based upon pools of tissue from four to five mice.
trality have been interpreted as showing that postnatal recruitment of brown adipocytes is not an ontogenetically determined process (31, 45). The similarity in developmental profiles for \( \text{Ucp1} \) during the in utero development of iBAT in A/J and B6 mice suggests that ontogenetic mechanisms are not variable genetically. Furthermore, the sustained similarities postnatally suggest that the inductive physiological response to reduced environmental temperature, during which the mass of iBAT increases \( \sim 5 \)-fold in both strains, is attributable to an amplification of processes initiated in utero. However, to our knowledge, an actual demonstration that permanent changes in the number of cells in the interscapular fat can be manipulated during early postnatal development through changes in the environmental temperature has not been reported. This raises the question of the ontogeny of brown adipocytes in white fat depots. In the mouse, most white fat is formed postnatally, and \( \text{Ucp1} \) mRNA is not detected until \( \sim 10 \) days of age, which is beyond the period of acute cold stress after birth and when animals have already developed a coat of fur. Accordingly, unlike iBAT, the appearance of brown adipocytes in RP does not coincide with the postpartum thermogenic requirements of the mouse. In addition, the strain difference in the expression of \( \text{Ucp1} \) in RP is very different between B6 and A/J mice, lasting only \( \sim 10 \) days in B6 mice and \( >1 \) month in A/J mice, during which no change in environmental temperature has occurred. These differences between iBAT and RP for expression of the brown adipocyte phenotype suggest that the developmental mechanisms for the two tissues are basically different. Why the timing of this process is so different between A/J and B6 mice is unknown. Additional factors that could be involved in establishing the morphology of white fat must include the neuroendocrine system and the establishment of systems of energy intake and expenditure.

Brown adipocytes may be inducible in adult human white fat depots (46). Therefore, it is plausible that a subpopulation of adipocytes that have been programmed as brown adipocytes in newborn humans may similarly revert to white adipocytes at a later stage of postnatal development. In adult humans, these white adipocyte-like cells may still have the potential to respond to adrenergic stimuli to again display their intrinsic brown adipocyte function and contribute to energy dissipation under conditions of positive energy balance.

The authors thank Christie Bearden for excellent technical assistance and Dr. Thomas Gettys for antibodies against UCP1 and PGC1\( \alpha \). The authors thank Dr. Barbara Gawronska-Kozak for photomicrography. This research was supported by National Institutes of Health Grants DK-58152 and P-30 DK072476.

REFERENCES

Brown adipocytes in white fat 51


