

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

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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.

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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

Abbreviations: ATF, activating transcription factor; B6, C57BL/6J; COXI, cytochrome c oxidase subunit I; CREB, cAMP response element binding protein; DIO2, type 2 T4 deiodinase; iBAT, interscapular brown adipose tissue; PGC1 α , peroxisome proliferator-activated receptor γ coactivator-1 α ; PKA, protein kinase A; PPAR, peroxisome proliferator-activated receptor; QTL, quantitative trait locus; RP, retroperitoneal fat; UCP1, uncoupling protein 1.

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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 β_3 -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 resistant 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 responsible for the phenotypic variation in obesity, and not iBAT. Based upon this rationale, our laboratory pursued a quantitative 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-1 α (*Pgc1 α*), and *Ppar α* , 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 differences in the mechanisms controlling the induction of the brown adipocyte phenotype that was established during early postnatal development, whereas induction of *Ucp1* in iBAT is simply a physiological response of mature differentiated brown adipocytes. A second related question concerns the origin of those adipocytes in RP that become differentiated brown adipocytes. There is morphological evidence that during the induction of brown

adipocytes in adult mice, cell division is essentially undetectable (23) and the induction of *Ucp1* reaches maximal 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 adipocyte differentiation from preadipocytes.

To address these questions, it is necessary to have basic information on the molecular features of the developmental 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 adipocytes 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 stimulation 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 *Ppar α* knockout mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at the Pennington Biomedical Research Center. All parental mice and their pups were kept at room temperature ($23 \pm 1^\circ\text{C}$) 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 gestation, 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 various measurements.

Real-time RT-PCR

Total RNA was isolated from tissues using Tri-Reagent (Molecular 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 previously (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 PPAR α , and pooled iBAT RNA from 18–20 day fetus was used as a standard for Preadipocyte factor 1. Standards for the remaining genes 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. 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-PGC1 α (1:4,000) polyclonal antibodies were kindly provided by Dr. Thomas Gettys (25). Rabbit anti-PPAR α (1:1,000) and anti-PPAR γ (1:1,000) polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies against protein kinase A (PKA) subunits (C α , RI α , RII α , pRII β , and RII β) 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 μ m 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 μ Ci/ μ g; 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 $^-$ released in tissue-free measurements. Dio2 activity was reported as fmol I $^-$ 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 \sim 10 days after birth and then both remained constant, although a

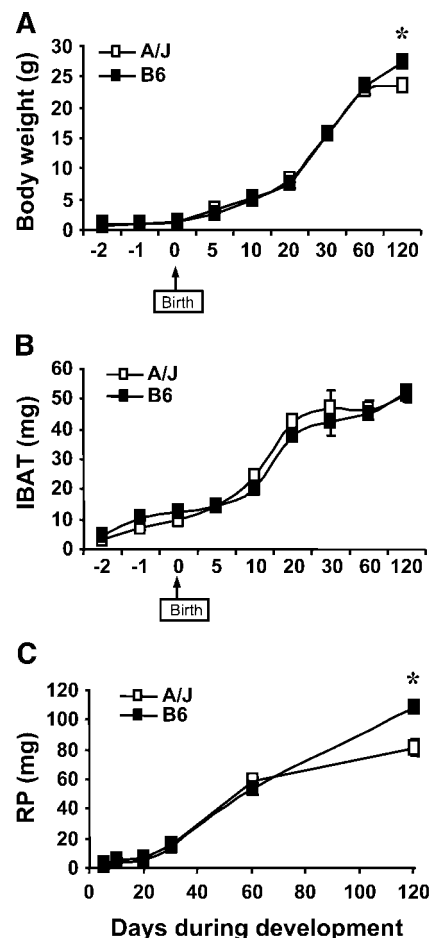


Fig. 1. Growth of A/J and C57BL/6J (B6) mice during prenatal and postnatal development. A, B: Body weight (A) and interscapular brown adipose tissue (iBAT) (B) weight measured 2 days before birth and up to 120 days of age. C: Retroperitoneal fat (RP) weight measured from 5 to 120 days of age. * $P < 0.05$ versus A/J mice. Each time point is based upon the analysis of 8–10 mice. Data were expressed as mean \pm SE. Differences between strains were analyzed for statistical significance by student's *t*-test.

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 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

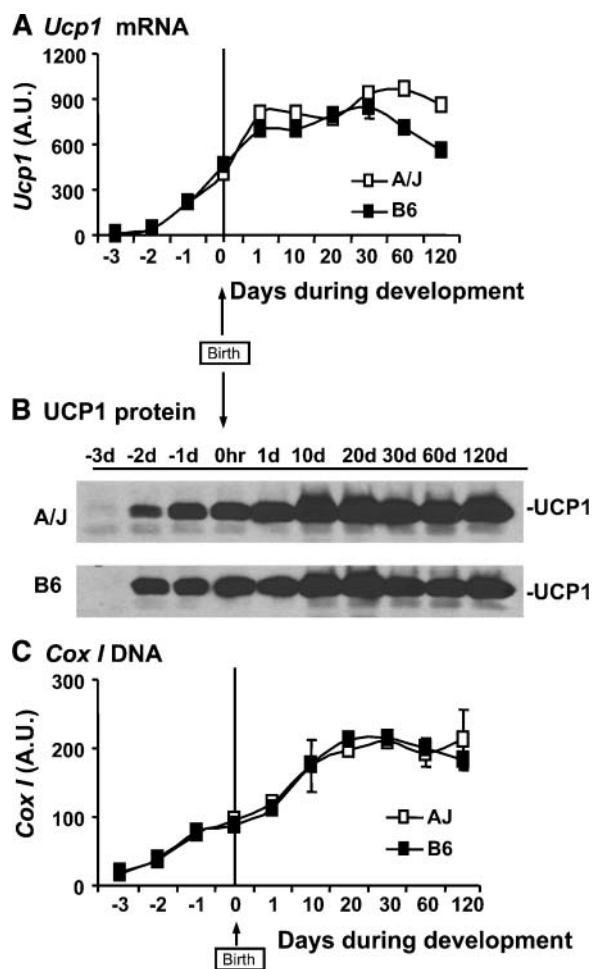


Fig. 2. Development of iBAT in A/J and B6 mice. Uncoupling protein 1 (*Ucp1*) mRNA (A), UCP1 protein (B), and mitochondrial DNA (C) expression in iBAT of A/J and B6 mice during prenatal and postnatal development. For *Ucp1* mRNA, each time point is the average value from four to nine individual animals 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.

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 an ~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**).

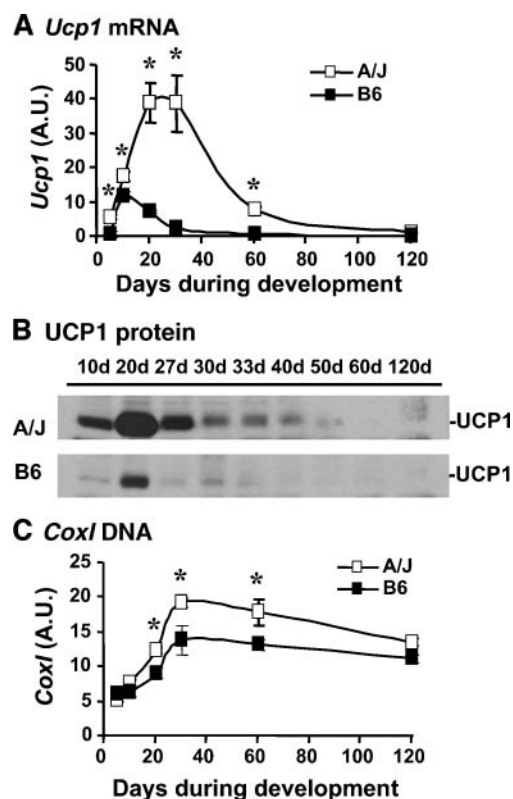


Fig. 3. Induction of *Ucp1* in RP of A/J and B6 mice during postnatal development. *Ucp1* mRNA (A), UCP1 protein (B), and mitochondrial DNA (C) expression. * $P < 0.05$ versus B6 mice. *Ucp1* mRNA data are based upon the analysis of three to six animals, and *CoxI* DNA data are based upon the analysis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

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 non-

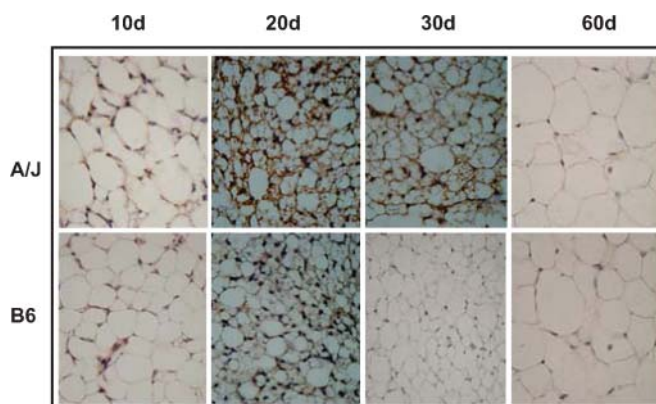


Fig. 4. Immunohistochemical assessment of the appearance of UCP1-positive multilocular brown adipocytes in RP of A/J and B6 mice during postnatal development. Magnification, $\times 20$.

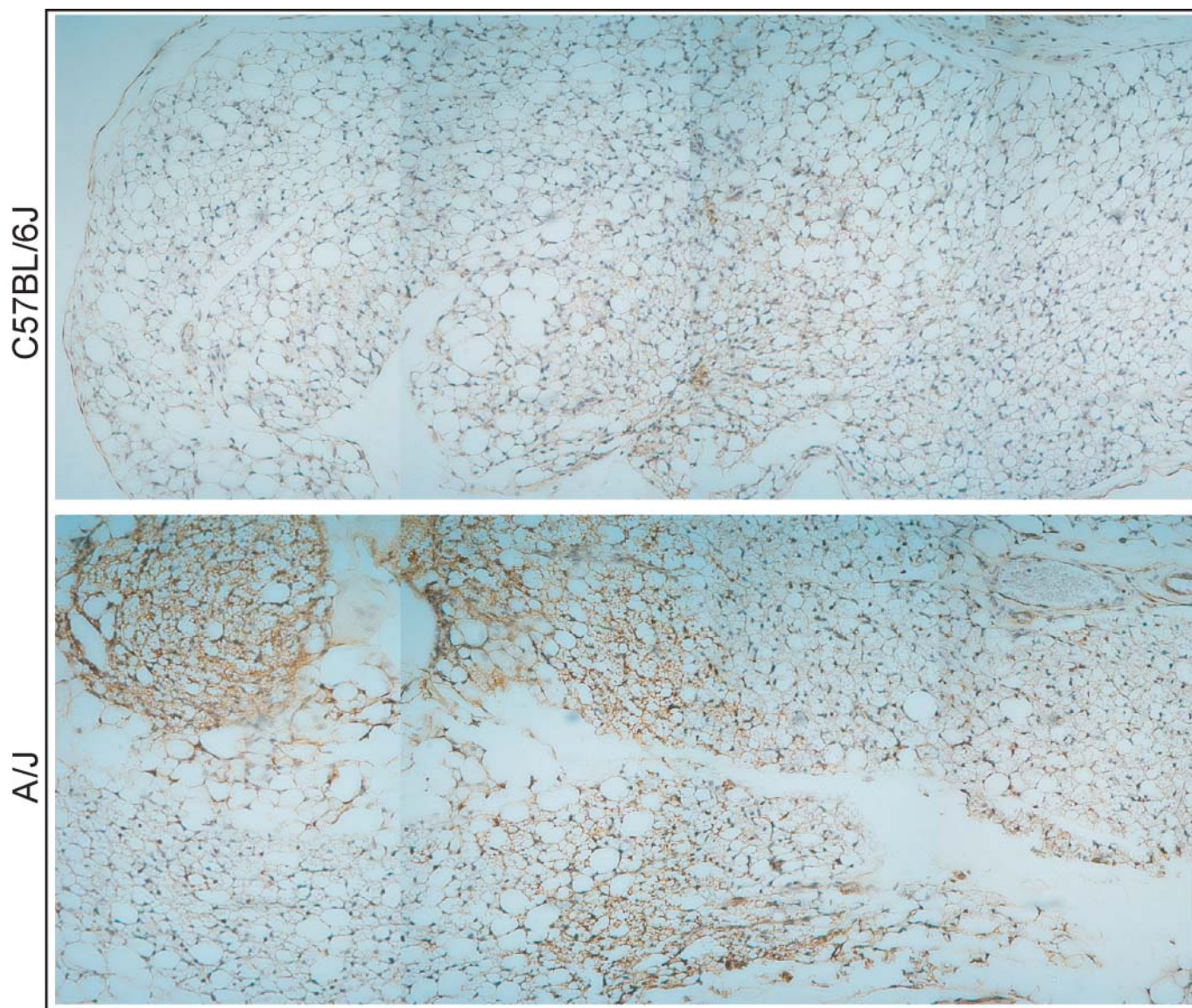


Fig. 5. A montage of photomicrographs of A/J and B6 RP immunohistograms stained with antibody against UCP1 and counterstained with hematoxylin. More than 80% of the RP depot is present in the figure. Magnification, 20 \times . (See supplementary data for patterns of staining in additional mice.)

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-

opment, 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 (23°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 *Ppar γ* , sterol-regulatory element binding protein 1c, *Cebp α* , $-\beta$, and $-\delta$, adipocyte P2 (*aP2*), *Pgc1 α* , and *Ppar α* ; 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 *CoxI* DNA (**Fig. 3C**), only *Pgc1 α* and *Ppar α* 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 *Ppar α* , comparable to the magnitude of *Ucp1* induction, suggested a significant role for *PPAR α* in the developmental regulation of RP remodeling. However, the induction of *Ucp1* in *Ppar α* 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 UCP1 were highly increased in A/J mice compared with B6 mice. In this set of tissues, differences in PGC1 α 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 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

		<i>Ucp1</i>		Relative Percent	
Tissue	Age/Conditions	A/J	B6	A/J	B6
<i>arbitrary units/cyclophilin mRNA</i>					
iBAT	20 days/23°C	773 ± 31 (n = 11)	789 ± 9 (n = 7)	100	102
	60 days/23°C	998 ± 27 (n = 3)	598 ± 58 (n = 6)	129	77
	60 days/4°C, 1 day	1,766 ± 27 (n = 4)	1,352 ± 28 (n = 3)	229	175
	60 days/4°C, 7 days	1,847 ± 73 (n = 5)	1,834 ± 70 (n = 3)	239	237
RP	20 days/23°C	63 ± 14 (n = 5)	6 ± 1.4 (n = 4)	8	0.8
	60 days/23°C	7 ± 1.4 (n = 5)	0.2 ± 0.04 (n = 6)	0.9	0.02
	60 days/4°C, 1 day	234 ± 63 (n = 5)	3 ± 1.6 (n = 3)	30	0.4
	60 days/4°C, 7 days	547 ± 59 (n = 5)	6 ± 1.0 (n = 3)	71	0.8

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

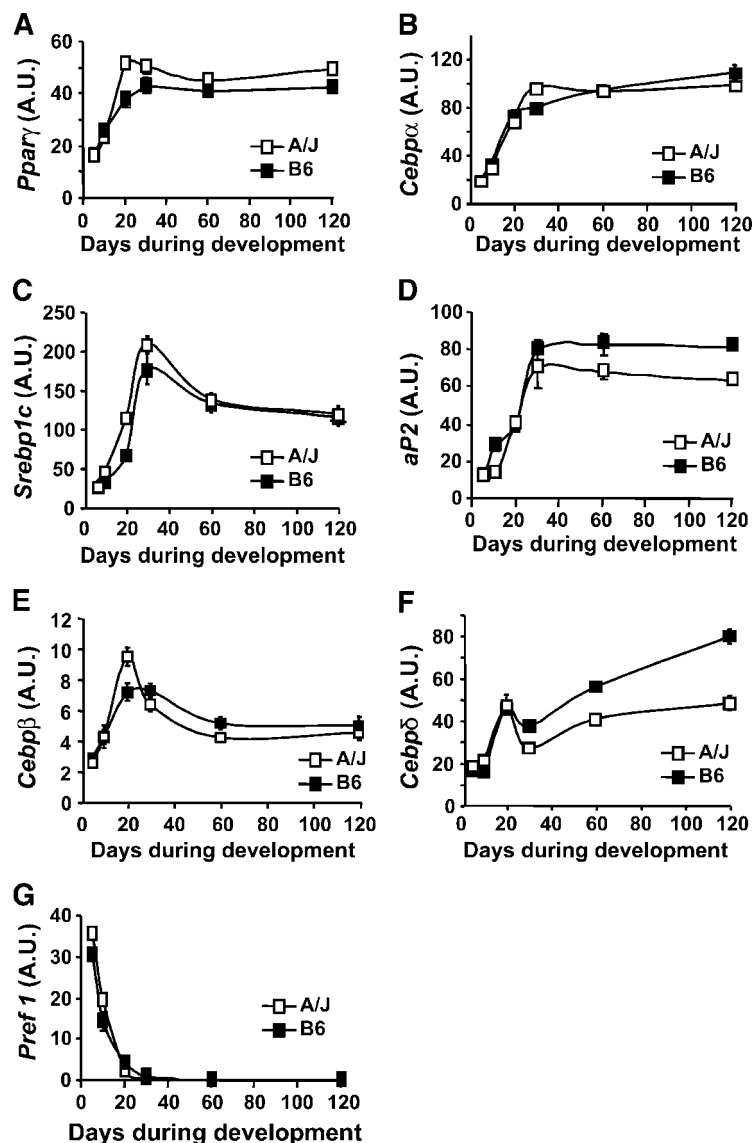


Fig. 6. Expression of the adipocyte and preadipocyte markers peroxisome proliferator-activated receptor γ (*Ppar γ* ; A), cAMP response element binding protein (*Cebp α* ; B), sterol-regulatory element binding protein 1c (*Srebp1c*; C), *aP2* (D), *Cebp β* (E), *Cebp δ* (F), and Preadipocyte factor 1 (*Pref1*; G) mRNA levels in RP during postnatal development and A/J and B6 mice. As in Fig. 3, each time point is based on the analysis of three to six mice assayed in duplicate and expressed relative to the levels of cyclophilin. A.U., arbitrary units.

pathways have been upregulated in A/J mice in a manner that could account for higher levels of *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 *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 *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-

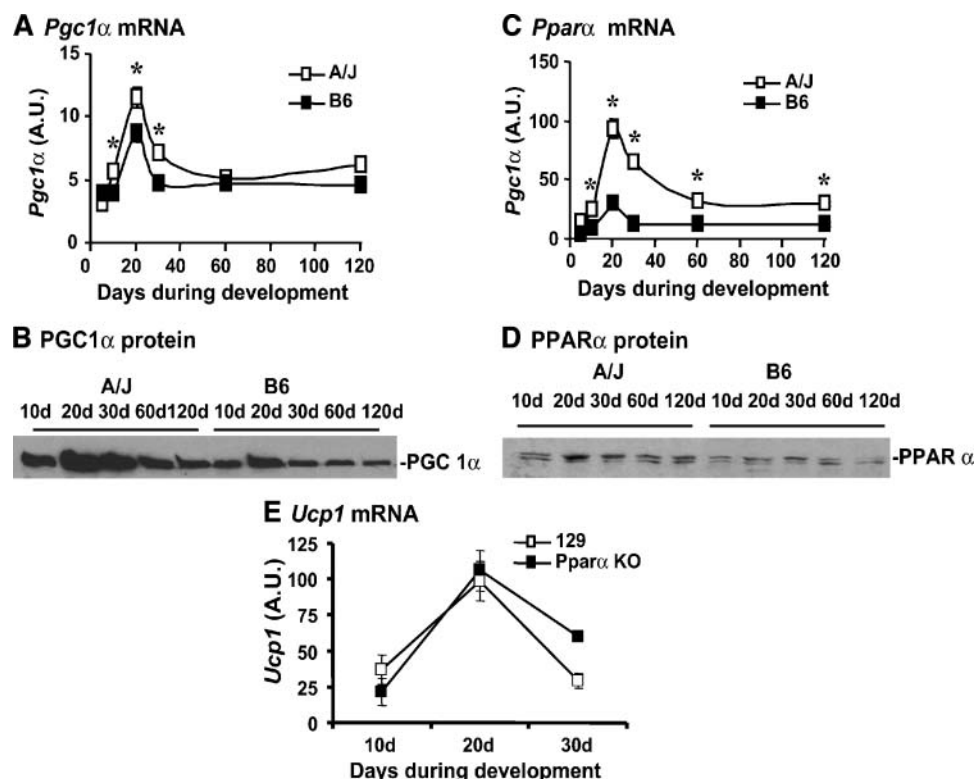


Fig. 7. Expression of peroxisome proliferator-activated receptor γ coactivator-1 α (*Pgc1 α*) and *Ppar α* in RP during postnatal development. A–D: *Pgc1 α* mRNA (A), PGC1 α protein (B), *Ppar α* mRNA (C), and PPAR α protein (D) levels in RP of A/J and B6 mice during postnatal development. E: *Ucp1* mRNA in RP of 129Sv/Im control and *Ppar α* knockout (KO) mice. Each time point for knockout mice contained four to six mice. A.U., arbitrary units.

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

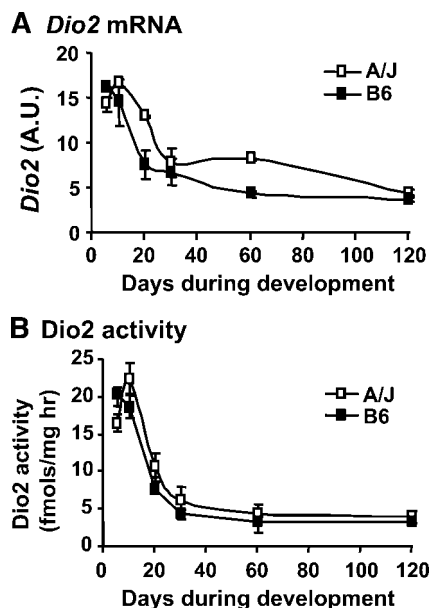


Fig. 8. Type 2 T4 deiodinase (*Dio2*) mRNA (A) and *Dio2* enzyme (B) activity in RP of A/J and B6 mice during postnatal development. Both *Dio2* mRNA and enzyme activity are based upon the analysis of three mice for each time point. A.U., arbitrary units.

variations in PGC1 α and PPAR α . 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 PPAR α 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 PPAR α 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 PPAR α is involved in *Ucp1* expression, the fact that the PPAR α 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 PPAR α 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-

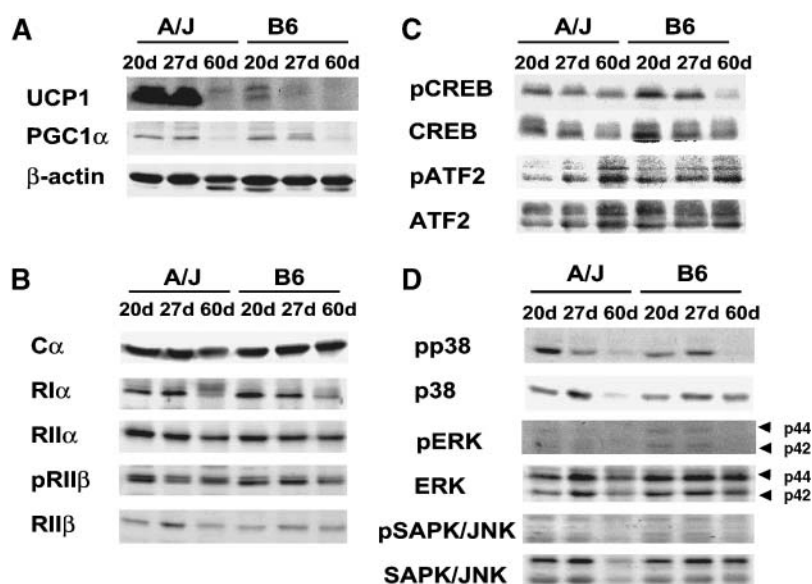


Fig. 9. Protein expression and phosphorylation patterns for UCP1 and cell signaling components in total tissue extracts of RP from A/J and B6 mice during postnatal development. A: UCP1 and PGC1 α expression. B: Catalytic and regulatory subunits of protein kinase A. C: CREB and activating transcription 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.

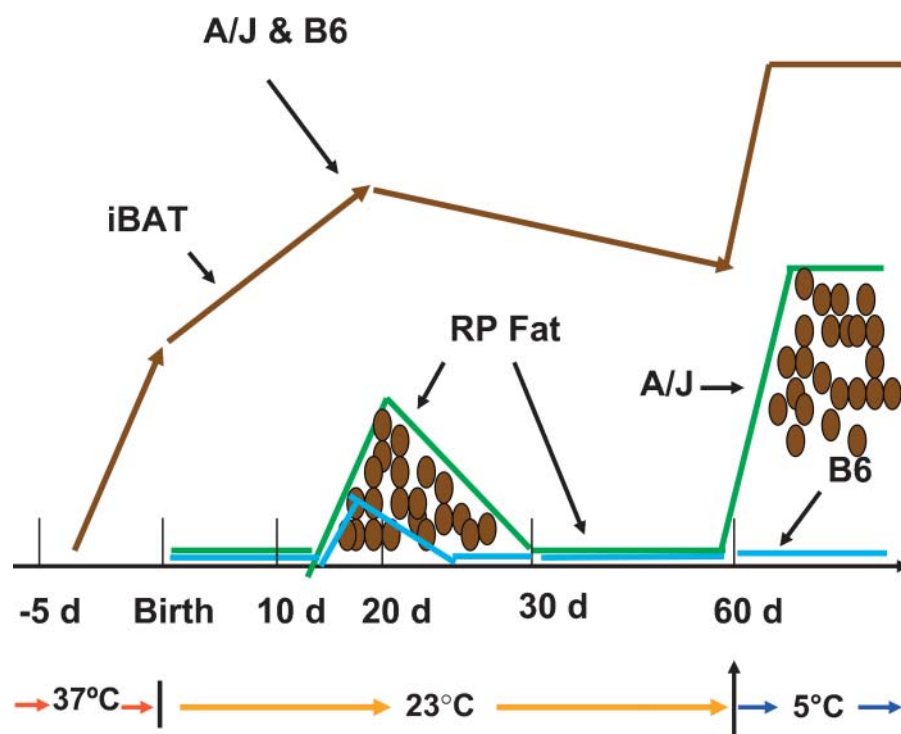


Fig. 10. A model illustrating the developmental profiles of brown adipocyte differentiation in A/J and B6 mice based upon mRNA, protein, and immunohistological analysis for *Ucp1*.

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 *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 ~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 *Ucp1* mRNA is not detected until ~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 *Ucp1* in RP is very different between B6 and A/J mice, lasting only ~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. **FIG**

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