Defective adipocyte differentiation in mice lacking the C/EBPβ and/or C/EBPδ gene

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To investigate the role of C/EBP family members during adipocyte differentiation in vivo, we have generated mice lacking the C/EBPβ and/or C/EBPδ gene targeting. Approximately 85% of C/EBPβ(–/–)δ(–/–) mice died at the early neonatal stage. By 20 h after birth, brown adipose tissue of the interscapular region in wild-type mice contained many lipid droplets, whereas C/EBPβ(–/–)δ(–/–) mice did not accumulate droplets. In addition, the epididymal fat pad weight of surviving adult C/EBPβ(–/–)δ(–/–) mice was significantly reduced compared with wild-type mice. However, these adipose tissues in C/EBPβ(–/–)δ(–/–) mice exhibit normal expression of C/EBPα and PPARγ, despite impaired adipogenesis. These results demonstrated that C/EBPβ and C/EBPδ have a synergistic role in terminal adipocyte differentiation in vivo. The induction of C/EBPα and PPARγ does not always require C/EBPβ and C/EBPδ, but co-expression of C/EBPα and PPARγ is not sufficient for complete adipocyte differentiation in the absence of C/EBPβ and C/EBPδ.

Keywords: adipogenesis/C/EBP family/PPARγ/ transcription factor

Introduction

There are two types of adipose tissue in the body, termed white adipose tissue (WAT) and brown adipose tissue (BAT). WAT stores excess energy as triglycerides and releases free fatty acids in response to energy requirements at other sites. BAT is involved in non-shivering and diet-induced thermogenesis through the uncoupling of oxidative phosphorylation, which is carried out by the action of a BAT-specific uncoupling protein (UCP). WAT is distributed in a number of locations throughout the body. In contrast, BAT is restricted to specific areas. WAT consists of adipocytes having a single fat droplet within the cells, whereas BAT has a multilocular disposition of fat droplets, i.e. a number of individual droplets within each adipocyte.

Adipocyte differentiation is a complex process involving a cascade of expression of many transcription factors and adipocyte-specific genes. Accumulating evidence suggests that two families of transcription factors, CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs), are important regulators of adipocyte gene expression and differentiation. The C/EBP family belongs to the large family of basic leucine zipper (bZip) transcription factors. All members of the C/EBP family have a C-terminal leucine zipper domain for dimerization and a basic domain for DNA binding, respectively. To date, six members have been cloned and characterized: C/EBPα, C/EBPβ (also known as NF-IL6, LAP, AGP/EBP, IL-6DBP or NF-M) (Akira et al., 1990; Chang et al., 1990; Descombes et al., 1990; Poli et al., 1990; Cao et al., 1991; Katz et al., 1993; Ness et al., 1993), C/EBPγ (Ig/EBP or GPE1BP), C/EBPδ (NF-IL6δ, CRP3 or CELF) (Cao et al., 1991; Kageyama et al., 1991; Williams et al., 1991; Kinoshita et al., 1992), C/EBPε (CRP1) and CHOP (gadd153). These proteins can both homodimerize and heterodimerize with each other and bind to the same C/EBP consensus sequences. Among them, three members of the C/EBP family, α, β and δ, are expressed in both WAT and BAT (Cao et al., 1991; Manchado et al., 1994; our unpublished data) and have been analyzed extensively with regard to their role in adipogenesis. Pre-adipocyte cell line 3T3-L1 cells differentiate into mature adipocytes when cultured in the presence of methylisobutylxanthine (MIX), dexamethasone (DEX), insulin (INS) and fetal bovine serum (FBS). These three C/EBP isoforms are expressed at specific periods during the differentiation of 3T3-L1 cells (Cao et al., 1991; Yeh et al., 1995). Ectopic expression of C/EBPα in 3T3-L1 pre-adipocytes blocked mitosis and induced adipocyte differentiation in the absence of hormonal stimulants. The blocking of C/EBPα induction in 3T3-L1 cells by antisense RNA inhibits adipocyte differentiation (Lin and Lane, 1992). Although NIH-3T3 fibroblasts cannot differentiate into adipocytes even in the presence of hormonal stimulants, forced expression of C/EBPα in NIH-3T3 cells results in adipose conversion in the absence of hormonal stimulants (Freytag et al., 1994; Lin and Lane, 1994). Recently, mice with a targeted disruption of the C/EBPα gene have been generated. These mice have a severely reduced mass of both BAT and WAT, demonstrating that C/EBPα is essential for in vivo adipocyte differentiation (Wang et al., 1995). Similarly, ectopic expression of C/EBPβ, and to a lesser extent of C/EBPδ, can convert NIH-3T3 cells into adipogenic cells even in the absence of hormonal stimulants (Yeh et al., 1995). Overexpression of a truncated and dominant-negative form of C/EBPβ inhibits adipocyte conversion of 3T3-L1 cells (Yeh et al., 1995). These results indicated that C/EBPβ and C/EBPδ are also involved in adipocyte differentiation.

PPARs consist of PPARα, γ and δ, and belong to the nuclear hormone receptor superfamily of ligand-activated transcription factors (Kliewer et al., 1994; Mangelsdorf,...
et al., 1995). Of these members, only PPARγ is expressed almost exclusively in adipose tissue (Chawla et al., 1994). Ectopic expression of PPARγ in NIH-3T3 fibroblasts leads to adipogenic conversion in the presence of its ligands thiazolidinedione or prostaglandin derivatives (Forman et al., 1995; Kliwer et al., 1995). When PPARγ and C/EBPα are expressed together ectopically in NIH-3T3 fibroblasts, they synergistically accelerate the conversion into adipocytes (Tontonoz et al., 1994b). Although the other two members are expressed to some extent in adipose tissue, PPARα is less adipogenic than PPARγ, and PPARδ cannot stimulate adipogenesis (Burn et al., 1996).

In the course of differentiation of adipocytes from 3T3-L1 pre-adipocytes, three C/EBP isoforms and PPARγ are expressed sequentially: C/EBPβ and δ are induced early and temporally in the process of differentiation, followed by PPARγ. C/EBPα is expressed at the late stage of differentiation (Cao et al., 1991; Yeh et al., 1995). A large body of evidence points to a model of a cascade of transcription factors involved in adipogenesis as follows. At the onset of differentiation, C/EBPβ and C/EBPδ are elevated in response to hormonal stimulation. Then, C/EBPβ and δ synergistically stimulate the expression of C/EBPα and PPARγ, both of which contain C/EBP-binding sites in their promoters (Christy et al., 1989; Cao et al., 1991; Wu et al., 1995, 1996; Zhu et al., 1995). C/EBPα activates several adipocyte-specific genes such as the stearyl-CoA desaturase (SCD1), insulin-responsive glucose transporter-4 (GLUT4), 422/aP2, phosphoenolpyruvate carboxykinase (PEPCK) and UCP genes which have functional C/EBP-binding sites in the promoter region (Christy et al., 1989; Park et al., 1990; Yubero et al., 1994).

PPARγ also transactivates the 422/aP2, PEPCK and lipoprotein lipase (LPL) genes whose promoters contain regulatory elements of PPARγ (Tontonoz et al., 1994a, 1995; Schoonjans et al., 1996). Furthermore, reciprocal gene activation between C/EBPα and PPARγ is demonstrated by the fact that ectopic expression of either transcription factor alone induces expression of the other. Therefore, at the final stage of adipocyte differentiation, PPARγ and C/EBPα may cooperatively induce adipogenic genes to establish the mature adipocyte phenotype.

However, this scenario is established mainly from in vitro experiments using pre-adipocyte and fibroblast cell lines, and it remains to be determined whether it is applicable to in vivo adipocyte differentiation. To investigate the in vivo role of C/EBPβ and C/EBPδ genes in adipocyte differentiation, we have generated C/EBPβ- and/or C/EBPδ-deficient mice by gene targeting. Our results demonstrated that C/EBPβ and C/EBPδ are essential for terminal differentiation of brown and white adipocytes in vivo.

Results

Generation of C/EBPβ and C/EBPδ knockout mice and C/EBPβ·C/EBPδ double knockout mice

Details as to the generation of C/EBPβ knockout mice have been described previously (Tanaka et al., 1995). Briefly, to construct the targeting vector for inactivation of the C/EBPβ gene, the DNA-binding domain and leucine zipper domain were deleted and replaced by a neomycin phosphotransferase (NEO) gene derived from pMC1Neo-poly(A) (Thomas and Cappelli, 1987) (Figure 1A). Five homologous recombinant clones were obtained by PCR screening and subsequent Southern blot analysis using probe A (Figure 1B). Genotypic analysis of progeny of intercrosses between heterozygotes is shown in Figure 1C.

To construct the targeting vector for disruption of the C/EBPδ gene, a Balb/c mouse genomic DNA library was screened using the human C/EBPδ cDNA as a probe (Kinosita et al., 1992). One clone containing the C/EBPδ gene, composed of one exon, was obtained. A 9.5 kb fragment spanning from 1 kb 5′ of the C/EBPδ transcriptional initiation site to 7.5 kb 3′ of the end of the exon was subcloned into pBluescriptII KS(+) plasmid vector. To inactivate the C/EBPδ gene, the promoter region, transcription start site and transactivation domain were deleted and replaced by NEO derived from pMC1Neo-poly(A) (Thomas and Cappelli, 1987). The MC1-herpes simplex virus thymidine kinase (HSV-TK) (Mansour et al., 1988) was ligated at the 3′ end of the homologous region.

The targeting vector was introduced into E14.1 embryonic stem cells by electroporation, and transfectants were selected with G418 and gancyclovir. Resistant clones were screened by PCR using primer pairs, one of which is located in the homologous 5′ region outside the targeting vector and the other is in the NEO gene (Figure 1A). Homologous recombination events were confirmed further by Southern blot analysis using probe B (Figure 1B). Two homologous recombinant clones were obtained from 342 G418- and gancyclovir-resistant colonies. These two clones were injected into C57BL/6 blastocysts, and chimeric mice were obtained. Both of these clones gave a germline transmission of mutant alleles. C/EBPδ homozygous (−/−) mice were obtained by intercrosses between C/EBPδ heterozygous (+/−) females and males (Figure 1C). C/EBPδ−/− mice of both sexes were fertile. However, C/EBPβ−/− females were infertile. To generate C/EBPβ·C/EBPδ double knockout mice, we first produced C/EBPβ−/+·C/EBPδ−/+ mutants by mating C/EBPβ−/+ mice with C/EBPδ−/+ mice. Then, these mice were crossed to C/EBPδ−/− to generate C/EBPβ−/+·C/EBPδ−/− mutants. C/EBPβ−/−·C/EBPδ−/− mice were finally obtained by intercrosses between C/EBPβ−/+·C/EBPδ−/− females and males. Northern blot analysis confirmed the absence of any detectable C/EBPβ mRNA in C/EBPβ−/− mice and C/EBPδ mRNA in C/EBPδ−/− mice (Figure 1D).

C/EBPβ·C/EBPδ double knockout mice died at the early neonatal stage

To determine the frequency of each of the knockout mice in heterozygote intercrosses, we analyzed the genotype of mice at 4 weeks of age. C/EBPδ−/− mice were obtained at the expected Mendelian ratio from C/EBPδ−/+ parents (Table 1), but both C/EBPβ−/+ offspring, from the cross of C/EBPβ−/+·C/EBPδ−/+ mutants, and C/EBPβ−/+·C/EBPδ−/− offspring, from C/EBPβ−/+·C/EBPδ−/− mutants, were obtained at a frequency lower than the expected Mendelian ratio (Table 1). The percentage of C/EBPβ−/− mice was 16.2%, while that of C/EBPβ−/+·C/EBPδ−/− mice was only 3.8%. Since genotype analysis at embryonic stages 15.5 and 18.5 d.p.c. revealed no embryonic death in either C/EBPβ−/− or C/EBPβ−/+·C/EBPδ−/− mice, we next
observed the fate of each newborn mouse. The initial survival of C/EBPβ(–/–) neonates was good; the percentage of C/EBPβ(–/–) mice at 10 h after birth was 22.4%, which is almost equal to the expected Mendelian ratio. Further observation of these neonates showed that ~35% of C/EBPβ(–/–) neonates died within 24 h after birth, with no further deaths thereafter (Table I). In the case of C/EBPδ(–/–)/C/EBPβ(–/–) mice, some had died at birth, as the percentage of these mice at 10 h after birth was only 13.0%, while others died within 24 h, a somewhat earlier time point than for C/EBPβ(–/–) mice. Consequently, 85% of double knockout mice died at the perinatal or early postnatal stage (Table I). However, C/EBPβ(–/–) and C/EBPδ(–/–)/C/EBPδ(–/–) neonates showed no gross abnormalities in appearance and could suckle.

**Lipid accumulation is impaired in interscapular BAT from C/EBPβ(–/–) and C/EBPδ(–/–) mice**

BAT is found only at certain sites in the body, such as in interscapular, perirenal and retroperitoneal regions, of which the interscapular BAT deposit is the largest. BAT develops during fetal life and becomes identifiable at the late fetal stage as a mass with small lobules. At the early stage of development, BAT is composed of only pre-adipocytes, where the lipid droplet distribution is dispersed. Subsequently, these cells store triglycerides and become multilocular, typical of brown adipocytes. Both C/EBPβ and C/EBPδ are expressed in BAT. We examined interscapular BAT microscopically in newborn wild-type, C/EBPβ(–/–), C/EBPδ(–/–) and C/EBPβ(–/–)/C/EBPδ(–/–) mice. As shown in Figure 2, interscapular BAT from wild-type mice had accumulated many lipid droplets by 20 h after birth. In contrast, lipid accumulation in the adipocytes of interscapular BAT from C/EBPβ(–/–) mice was significantly reduced compared with that from wild-type mice. Lipid accumulation in C/EBPδ(–/–) BAT was only slightly impaired. Interestingly, C/EBPβ(–/–)/C/EBPδ(–/–) BAT could not accumulate fat droplets (Figure 2A).

Adipose tissue is composed of several types of cells that are at different stages of differentiation. Pre-adipocytes are defined as cells with no lipid accumulation in the
cytoplasm. Pre-adipocytes differentiate into immature adipocytes which contain only small lipid droplets in the cytoplasm, and mature adipocytes whose cytoplasms are filled with well-developed lipid droplets (Aihaad et al., 1992). To evaluate the extent of differentiation of each adipocyte in this tissue in more detail, we stained lipid components in the adipocytes with Oil-Red-O, which is significantly reduced as compared with that of wild-type mice (Figure 2B). However, the majority is almost the same or somewhat reduced compared with that of wild-type mice (Figure 2B). This suggested that differentiation of the cells in C/EBPβ(–/–) adipose tissue is arrested while the adipocyte is immature. The intracytoplasmic lipid content of C/EBPδ(–/–) adipocytes is almost the same or somewhat reduced compared with that of wild-type mice (Figure 2B). However, the majority of the cells in C/EBPβ(–/–)δ(–/–) BAT contain no lipid droplets, although some cells have one or two droplets in the cytoplasm (Figure 2B). This finding suggests that the differentiation is blocked at the pre-adipocyte stage in the majority of the cells of interscapular BAT from C/EBPβ(–/–)δ(–/–) mice.

**Decayed expression of UCP1 but not C/EBPα and PPARγ in interscapular BAT from C/EBPβ(–/–)δ(–/–) mice**

A number of genes are expressed specifically in adipose tissue. It is suggested that the timing of the expression of these genes is essential for the acquisition and maintenance of adipocyte phenotype. LPL is expressed at the early stage of differentiation, whereas expression of aP2 and PEPCK is detected during the late or terminal stage of differentiation. UCP1 is the functional marker of terminally differentiated brown adipocytes. To examine the expression of these adipocyte-specific genes, we performed Northern blot analysis of RNA isolated from BAT with several adipogenic cDNAs as probe. RNA was extracted from interscapular fat of 18.5 d.p.c. mice embryos to exclude the effect of differences of feeding. As shown in Figure 3A, UCP1 mRNA expression in C/EBPβ(–/–)δ(–/–) BAT was markedly reduced, and was almost undetectable in two of three C/EBPβ(–/–)δ(–/–) embryos. Figure 3B shows a densitometric analysis of Figure 3A. Densitometric values are normalized to β-actin, and each bar represents the mean ± SD of three independent pairs of embryos. UCP1 expression in C/EBPβ(–/–), C/EBPδ(–/–) and C/EBPβδ(–/–)δ(–/–) BAT was 31.2, 46.2 and 8.7%, respectively. aP2 mRNA expression is slightly reduced in C/EBPβ(–/–)δ(–/–) BAT (52.3% of wild-type embryo). PEPCK and LPL expression was indistinguishable between wild-type and the three mutant embryos.

We further analyzed the expression of C/EBPα and PPARγ, the transcription factors whose expression is shown to be induced by C/EBPβ and C/EBPδ. In contrast to the hypothesis of previous studies using in vitro culture systems, the expression of C/EBPα and PPARγ in interscapular BAT of C/EBPβ(–/–), C/EBPδ(–/–) and C/EBPβδ(–/–)δ(–/–) mice is comparable with that in wild-type mice (Figure 3B). This finding shows that C/EBPα and PPARγ can be induced in vivo in the absence of C/EBPβ and C/EBPδ expression.

**Embryonic fibroblasts from C/EBPβ(–/–)-/C/EBPδ(–/–) mice could not differentiate into adipocytes**

To demonstrate that these phenotypes are not a secondary effect of systemic growth defects but rather the primary effect of a lack of C/EBPβ and C/EBPδ in adipocytes and to investigate the difference in the results obtained from the present analyses of knockout mice and previous in vitro culture systems, we prepared primary embryonic fibroblasts from each knockout mouse and examined their differentiation properties, since cell lines frequently used for in vitro adipocyte differentiation such as 3T3-L1 and 3T3-F442A pre-adipose cells are derived from embryonic fibroblasts. These cell lines can differentiate into adipocytes in the presence of MIX, DEX, INS and FBS (standard differentiation induction medium). The embryonic fibroblasts from each knockout mouse were cultured to confluence and then treated with the standard differentiation induction medium. As shown in Figure 4A, embryonic fibroblasts from wild-type mice differentiated into adipocytes and accumulated many lipid droplets in response to hormonal stimulants. The differentiation frequency of embryonic fibroblasts from C/EBPβ(–/–) mice is significantly reduced as compared with that of wild-type embryonic fibroblasts. C/EBPδ(–/–) embryonic fibroblasts exhibited slightly reduced differentiation. In contrast, C/EBPβδ(–/–)δ(–/–) embryonic fibroblasts can hardly differentiate into adipogenic cells and accumulate very few lipid droplets. Low magnitude observations with Oil-Red-O staining demonstrated clear differences in adipocyte phenotype (Figure 5B).

There are specific marker genes for each stage of adipocyte differentiation. For example, LPL and fatty acid

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**Table I. Genotype of progeny of heterozygote intercrosses**

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<tr>
<th>Stage</th>
<th>Genotype</th>
<th>β (+/+)</th>
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<td>C/EBPβ(–/–)×C/EBPβ(+/+) Stage</td>
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<td>δ (+/+)</td>
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transporter are expressed at the pre-adipocyte stage and are the earliest markers of differentiation, while aP2 and GLUT4 are expressed at the immature adipocyte stage, and PEPCK and adipsin at the mature stage (Ailhaud et al., 1992). These findings were based mainly on in vitro experiments using a 3T3-L1 pre-adipocyte differentiation system. We next examined the expression of adipocyte-specific genes during the course of differentiation. Adipo-
appeared to be slightly impaired in differentiation, but were considered to mature as normal because they expressed PEPCK, a very late marker of differentiation. 5,8,11,14-Eicosatetraenoic acid (ETYA) is an activator of PPARγ and can accelerate PPARγ-mediated adipogenesis in certain culture conditions. However, ETYA did not affect the differentiation in our system (data not shown). These findings indicate that C/EBPβ and C/EBPδ synergistically accelerate adipocyte differentiation, and ablation of both of these genes leads to almost complete inhibition of adipocyte differentiation.

We examined the expression of C/EBPα and PPARγ. C/EBPα expression was reduced markedly in C/EBPβ(–/–) δ(–/–) embryonic fibroblasts, although C/EBPα expression in C/EBPβ(–/–) or C/EBPδ(–/–) fibroblasts was comparable with that of wild-type fibroblasts. PPARγ expression was reduced slightly in C/EBPδ(–/–) fibroblasts and decreased markedly in C/EBPβ(–/–) and C/EBPβδ(–/–) δ(–/–) fibroblasts.

Taken together, adipocyte differentiation both in interscapular BAT and in cultured embryonic fibroblasts treated with adipogenic hormones was apparently blocked, but the expression of adipocyte-specific genes and of adipogenic transcription factors was quite different; in embryonic fibroblasts derived from C/EBPβ(–/–)δ(–/–) mice, the expression of genes induced following the expression of C/EBPβ and C/EBPδ genes was severely impaired. In contrast, in the case of interscapular BAT, transcription factors such as C/EBPα and PPARγ and the late differentiation markers, aP2, PEPCK and adipins, but not UCP1, were induced normally.

**Development of epidydimal WAT in C/EBPβ(–/–)δ(–/–) mice**

Approximately 85% of C/EBPβ(–/–)δ(–/–) mice died within 24 h after birth. The few mice that survived beyond this period were of normal appearance and exhibited a normal rate of mortality in adulthood. WAT cannot be detected macroscopically during embryogenesis and at birth, and is considered to function mainly as an energy store and in the regulation of metabolism in later life. We examined the abnormalities of WAT in surviving adult mice. WAT can be detected in epidydimal, parametrial, inguinal and subcutaneous regions within the body. Since it is plentiful and can be dissected easily from the surrounding tissues, we prepared epidydimal WAT from each mouse, and measured its weight. As shown in Figure 6A, the epidydimal WAT weight of C/EBPβ(–/–)δ(–/–) mice is significantly lower than that of wild-type mice (~30% of wild-type). The WAT weight of C/EBPβ(–/–) or C/EBPδ(–/–) mice tended to be lower, but not significantly so. Reduced WAT volume is not due to the secondary effect of systemic growth retardation, since the body weight values of these mice were not significantly different (data not shown). Figure 6B shows the appearance of representative epidydimal WAT from wild-type and C/EBPβ(–/–)δ(–/–) mice. Histologically, adipocytes in epidydimal WAT from C/EBPβ(–/–)δ(–/–) mice were normal, with a typical unilocular or signet-ring appearance (data not shown).

We examined the expression of adipocyte-specific genes in epidydimal WAT. As shown in Figure 7A, there were no differences in the expression of LPL and aP2 between wild-type, C/EBPβ(–/–), C/EBPδ(–/–) and C/EBPβδ(–/–) cells.
Fig. 4. Adipogenic differentiation from primary embryonic fibroblasts. Primary embryonic fibroblasts from each knockout mouse were cultured in the presence of standard differentiation induction medium (containing 0.5 mM MIX, 1 μM DEX, 5 μg/ml INS and 10% FBS). After 8 days of differentiation, cells were observed by light microscopy with (B) or without (A) Oil-Red-O staining. Cells from wild-type (upper left column), C/EBPβ(−/−) (upper right column), C/EBPδ(−/−) (lower left column) and C/EBPβ(−/−)·C/EBPδ(−/−) mice (lower right column) are shown. The original magnification is ×10 for (A) and ×20 for (B). This experiment was repeated five times using cells prepared from five different embryos, and similar results were obtained.

δ(−/−) mice. C/EBPα and PPARγ were also expressed in C/EBPβ(−/−), C/EBPδ(−/−) and C/EBPβ(−/−)·δ(−/−) mice to an extent similar to that in wild-type mice. These findings suggest that maturation of epidydimal adipocytes of C/EBPβ(−/−)·δ(−/−) mice is almost normal, despite a reduced volume of epidydimal WAT.
neonatal development

C/EBP

depot in inguinal WAT. This phenotype resembles that of accumulation in interscapular BAT and no detectable lipid mice died within 8 h of birth. These mice had little lipid mortalities of liver and lung were detected in C/EBP

This defect may be the major cause of neonatal death of

mice (Flodby et al., 1993). When diphtheria toxin A chain indicated.

RNA were subjected to Northern blot analysis using the probes as indicated.

Discussion

Remarkable progress has been made recently in elucidating the molecular basis of adipose differentiation. The process involves the sequential expression of transcription factors, C/EBPβ, C/EBPδ, PPARγ and C/EBPα. However, this scenario is based mainly on cultured cell systems in which adipogenic hormones can induce mature differentiation of pre-adipocyte cell lines. In vivo, adipocyte development is not fully understood. In this study, we have generated mice lacking C/EBPβ and/or C/EBPδ, and analyzed the in vivo role of these transcription factors in adipocyte differentiation.

C/EBPβ and C/EBPδ genes are essential for neonatal development

Unexpectedly, 35% of C/EBPβ(−/−) mice and 85% of C/EBPβ(−/−)·δ(−/−) mice died at the early neonatal stage. In the case of C/EBPα gene targeting, C/EBPα knockout mice died within 8 h of birth. These mice had little lipid accumulation in interscapular BAT and no detectable lipid depot in inguinal WAT. This phenotype resembles that of C/EBPβ(−/−)·δ(−/−) mice. In addition, C/EBPα(−/−) mice showed severe hypoglycemia due to the defective induction of liver enzymes essential for glucose metabolism. This defect may be the major cause of neonatal death of C/EBPα(−/−) mice. Furthermore, histological abnormalities of liver and lung were detected in C/EBPα(−/−) mice (Flodby et al., 1996). C/EBPβ(−/−), C/EBPδ(−/−) and C/EBPβ(−/−)·δ(−/−) mice showed no histological abnormalities in these tissue and did not suffer from hypoglycemia (data not shown). These findings suggest that the cause of neonatal death of C/EBPβ(−/−)·δ(−/−) mice is quite different from that of C/EBPα(−/−) mice. Interestingly, transgenic ablation of BAT in mice leads to early neonatal death (Ross et al., 1993). When diphtheria toxin A chain is expressed specifically in adipose cells using the aP2 gene promoter, transgenic mice with high levels of toxin expression died within 24 h of birth, although mice with low expression survived. The expression of the UCP1 gene in interscapular BAT of these transgenic mice was decreased in accordance with the expression of transgene. These results suggest that thermogenic activity in BAT is essential for the survival of newborn mammals. The high mortality of C/EBPβ(−/−)·δ(−/−) mice may be due to the defective differentiation of BAT and its resulting dysfunction as a thermogenic organ. The marked decrease of UCP1 gene expression in C/EBPβ(−/−)·δ(−/−) BAT may support this speculation. However, UCP1 itself is not an essential factor for adipocyte development and thermogenic activities in neonates because UCP1 knockout mice suffered neither neonatal death nor impaired adipocyte differentiation of interscapular BAT (Enerbäck et al., 1997). Other adipocyte-specific factors that are regulated by C/EBPβ or/and C/EBPδ may be essential for neonatal survival. Further study such as the generation of mice lacking C/EBPβ and C/EBPδ in an adipose tissue-specific manner will be required to clarify this point.

Defects in adipocyte maturation in C/EBPβ(−/−)·δ(−/−) mice despite normal expression of C/EBPα and PPARγ

Previous experiments proposed that adipocyte development is regulated by many transcription factors which are expressed sequentially. C/EBPβ and C/EBPδ are expressed early on in differentiation and synergistically activate the transactivation of both the PPARγ and C/EBPα genes. Then both PPARγ and C/EBPα directly induce adipogenic genes, which results in the adipocyte phenotypes (Cao et al., 1991; Yeh et al., 1995; Wu et al., 1996). Indeed, many adipogenic genes harbor the DNA-binding sites for both PPARγ and C/EBPα. If this hypothesis is true, the expression of PPARγ and C/EBPα and the subsequent induction of adipogenic marker genes would be markedly reduced in C/EBPβ(−/−)·δ(−/−) mice. In the in vitro study of adipocyte differentiation using primary embryonic fibroblasts derived from C/EBPβ(−/−)·δ(−/−) mice, the expression of both PPARγ and C/EBPα was severely reduced to almost undetectable levels, and the expression of several adipogenic marker genes was also impaired in line with defective lipid accumulation, which agrees with the above hypothesis. However, in vivo, these transcription factors were expressed normally in neonatal BAT of C/EBPβ(−/−)·δ(−/−) mice despite the defective lipid accumulation. This finding demonstrated that PPARγ and C/EBPα can be induced in the absence of C/EBPβ and δ. In vivo differentiation experiments, the only differentiation stimuli are MIX, DEX, INS and FBS; however, in vivo, there are many other adipogenic factors such as growth hormone, IGF-1, prostaglandins and β3-adrenergic ligand. With the restricted number of stimuli in vitro, PPARγ and C/EBPα are induced according to a proposed cascade of transcription factors, but in vivo, C/EBPα and PPARγ may be induced by other transcription factors. It is important to identify such transcription factors for alternative pathways that lead to C/EBPα or PPARγ expression.

Despite normal expression of PPARγ and C/EBPα, C/EBPβ(−/−)·δ(−/−) mice have almost the same phenotype as C/EBPα(−/−) mice in terms of interscapular BAT, i.e.

![Fig. 5. Northern blot analysis of primary embryonic fibroblasts during adipogenic differentiation. Primary embryonic fibroblasts from each knockout mouse were differentiated in the presence of the hormonal stimulants described in Materials and methods. RNA was extracted from these cells at day 0, 2, 5 and 8 of differentiation. Ten μg of total RNA were subjected to Northern blot analysis using the probes as indicated.](image-url)
impairment of lipid accumulation and a reduced level of UCP1 expression. Interestingly, C/EBPβ and C/EBPδ were expressed normally in C/EBPα(−/−) mice. These findings indicate that loss of C/EBPα function cannot be compensated by C/EBPβ and C/EBPδ, and vice versa.

The expression of adipogenic marker genes, with the exception of UCP1, was almost normal, despite defective adipocyte maturation in C/EBPβ(−/−)δ(−/−) BAT. Furthermore, mice lacking the aP2 gene exhibit normal adipocyte development (Hotamisligil et al., 1996) and the mass of interscapular BAT in mice lacking UCP1 is significantly greater with increased deposition of triglyceride in each adipocyte (Enerbäck et al., 1997). These findings indicate that the expression of the adipogenic marker genes is not associated with the maturity of adipocyte differentiation.

**Decreased WAT volume in C/EBPβ(−/−)δ(−/−) mice**

WAT is the organ that stores energy as triglycerides. Excess energy storage in fat tissue leads to obesity, and increased fat volume is a hallmark of this. Mild obesity reflects an increase in the size of each adipocyte (hypertrophic obesity), while more severe obesity or obesity arising in childhood typically involves increased fat cell number (hyperplastic obesity) (Spiegelman and Flier, 1996). Since hyperplastic obesity is closely related to many clinical diseases such as coronary heart disease, non-insulin-dependent diabetes mellitus, hypertension and hyperlipidemia, controlling the adipose cell number in the body may lead to the prevention of such diseases. An intriguing finding is the decreased epidydimal WAT weight in C/EBPβ(−/−)δ(−/−) mice, despite normal histology and normal expression of the adipogenic genes. Since individual C/EBPβ(−/−)δ(−/−) mice have variations in the expression of adipogenic genes in BAT, as shown in Figure 3, the fact that only a few C/EBPβ(−/−)δ(−/−) mice survived to adulthood implies that surviving knockout mice exhibit a somewhat mild phenotype. Nevertheless,
adult C/EBPβ(−/−)δ(−/−) mice exhibit a decreased weight of epidydimal WAT. This is considered to be due to the reduced number of adipocytes in C/EBPβ(−/−)δ(−/−) WAT, since epidydimal WAT of C/EBPβ(−/−)δ(−/−) mice is histologically normal and the size of each adipocyte differs little from that of wild-type mice (data not shown). This suggested that C/EBPβ and δ are involved in the increase in the number of adipocytes after birth, and that inhibition of C/EBPβ and C/EBPδ at the appropriate times may serve as a treatment for obesity.

**Conclusion**

Our present study demonstrated that C/EBPβ and C/EBPδ can synergistically promote terminal adipocyte differentiation both in vitro and in vivo. The defects of adipocyte differentiation observed in C/EBPβ(−/−)δ(−/−) mice are summarized in Table II. In in vitro differentiation systems using primary embryonic fibroblasts, cells from C/EBPβ(−/−)δ(−/−) mice neither differentiate into mature adipocytes nor express C/EBPα and PPARγ. This suggests that adipocyte differentiation in vitro proceeds according to the proposed transcriptional regulatory cascade in which adipogenic transcription factors such as C/EBP family members and PPARs are activated sequentially. However, in vivo, C/EBPα and PPARγ can be induced without expression of C/EBPβ and C/EBPδ. In addition, despite normal expression of C/EBPα or PPARγ, adipogenesis is severely impaired in C/EBPβ(−/−)δ(−/−) mice. These findings demonstrated that the induction of C/EBPα and PPARγ does not always require C/EBPβ and C/EBPδ, but co-expression of C/EBPα and PPARγ is not sufficient for complete adipocyte differentiation in their absence. Further studies to identify the target genes that are regulated by C/EBPβ and C/EBPδ using our knockout mice and embryonic fibroblasts derived from them will produce a clearer picture of adipocyte differentiation.

**Materials and methods**

**Construction of targeting vector**

Construction of the targeting vector for disruption of the C/EBPδ gene was as described (Tanaka et al., 1995).

To construct the targeting vector for disruption of the C/EBPδ gene, a Balb/c mouse genomic DNA library, packaged in EMBL3 (Clontech), was screened using the human C/EBPδ cDNA as a probe (Kinoshita et al., 1992). One clone containing the C/EBPδ gene, composed of one exon, was isolated. A 9.5 kb genome fragment spanning from 1 kb 5’ of the C/EBPδ transcription initiation site to 7.5 kb 3’ of the exon was subcloned into pBluescriptII KS+(+) plasmid vector. To inactivate the C/EBPδ gene, a 0.8 kb fragment containing the promoter region, transcription start site and transactivation domain was deleted and replaced by a 1.1 kb Xhol–SalI fragment of NEO derived from pMC1Neo-pol(A) (Thomas and Cappechi, 1987). Homologous regions 5’ and 3’ of NEO were 1.1 and 7.7 kb, respectively. For negative selection against random integration, the MCI1-HSV-TK (Mansour et al., 1988) was ligated at the 3’ end of the homologous region.

**ES cell culture and generation of mutant mice**

The strategy for culturing and electroporation of E14-1 embryonic stem cells and screening of homologous recombinant clones was as described.

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**Table II. Summary of defects in adipocyte differentiation observed in C/EBPβ(−/−)δ(−/−) mice**

<table>
<thead>
<tr>
<th>Cell or tissue</th>
<th>Embryo</th>
<th>Newborn</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell or tissue</td>
<td>primary embryonic fibroblasts</td>
<td>interscapular BAT</td>
<td>epidydimal WAT</td>
</tr>
<tr>
<td>in vitro differentiation system</td>
<td>in vivo</td>
<td>in vivo</td>
<td></td>
</tr>
<tr>
<td>Defects</td>
<td>blockade of differentiation from pre-adipocytes to adipocytes</td>
<td>severely reduced lipid accumulation</td>
<td>reduced volume of fat pad</td>
</tr>
<tr>
<td>Expression of</td>
<td>C/EBPα↓</td>
<td>C/EBPα→</td>
<td>C/EBPα→</td>
</tr>
<tr>
<td>transcription</td>
<td>PPARγ↓</td>
<td>PPARγ→</td>
<td>PPARγ→</td>
</tr>
<tr>
<td>factors</td>
<td>LPL→</td>
<td>LPL→</td>
<td>LPL→</td>
</tr>
<tr>
<td>Expression of</td>
<td>aP2↓</td>
<td>aP2→ or δ↓</td>
<td>aP2→</td>
</tr>
<tr>
<td>adipogenic</td>
<td>PEPCK↓</td>
<td>PEPCK→</td>
<td>PEPCK→</td>
</tr>
<tr>
<td>marker genes</td>
<td>UCP1↓</td>
<td>UCP1↓</td>
<td>UCP1↓</td>
</tr>
</tbody>
</table>

Comments: The expression pattern of transcription factors and adipogenic marker genes agrees with the cascade model of adipocyte differentiation previously proposed.
Preparation of primary embryonic fibroblasts and induction of adipogenic differentiation

Primary embryonic fibroblasts were harvested from 14.5 d.p.c. embryos. Cells were cultured at 37°C in αMEM; Gibco/BRL supplemented with 10% heat-inactivated FBS (Hyclone). Cells were plated to 24-well or 60 mm plastic dishes and propagated to confluence. Two days later, medium was replaced with standard differentiation induction medium containing 0.5 mM MIX (Sigma), 1 μM DEX (Sigma), 5 μg/ml INS (Sigma) and 10% FBS (Hyclone). This medium was renewed every other day. After 8 days, the appearance of cytoplasmic lipid accumulation was observed by bright-field microscopy with or without Oil-Red-O staining. The Oil-Red-O staining was performed as follows; cells were washed with phosphate-buffered saline (PBS), and then stained with 60% filtered Oil-Red-O stock solution [0.15 g of Oil-Red-O (Sigma) in 50 ml of isopropanol] for 30 min at 37°C. Cells were washed first with 60% isopropanol and then briefly with water and visualized. To prepare RNA for Northern blotting, cells were harvested at days 0, 2, 5 and 8 of differentiation.

Histological analysis

At 20 h after birth, neonates were fixed separately in Bouin’s reagent or 20% formaldehyde in PBS. Genomic DNA was prepared from the tail of each neonate, and PCR analysis was performed to determine the genotype. After PCR analysis, mice with the desired genotype were subjected to the following steps. For HE staining, neonates, which were fixed in Bouin’s reagent, were embedded in paraffin, sectioned transversely at 2 μm intervals, and stained with hematoxylin and eosin. For fat staining, neonates, which were fixed in 20% formaldehyde in PBS, were frozen, sectioned transversely at 10 μm intervals, and stained with Oil-Red-O. All sections were examined by light microscopy.

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Targeting of C/EBP\(\beta\) and C/EBP\(\delta\)


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