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Acyl-CoA Binding Protein Gene Ablation Induces Pre-implantation Embryonic Lethality in Mice

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Abstract

Unique among the intracellular lipid binding proteins, acyl-CoA binding protein (ACBP) exclusively binds long-chain fatty acyl-CoAs (LCFA-CoAs). To test if ACBP is an essential protein in mammals, the ACBP gene was ablated by homologous recombination in mice. While ACBP heterozygotes appeared phenotypically normal, intercrossing of the heterozygotes did not produce any live homozygous deficient (null) ACBP^(-/-) pups. Heterozygous and wild type embryos were detected at all post-implantation stages, but no homozygous ACBP-null embryos were obtained—suggesting that an embryonic lethality occurred at a pre-implantation stage of development, or that embryos never formed. While ACBP-null embryos were not detected at any blastocyst stage, ACBP-null embryos were detected at the morula (8-cell), cleavage (2-cell), and zygote (1-cell) pre-implantation stages. Two other LCFA-CoA binding proteins, sterol carrier protein-2 (SCP-2) and sterol carrier protein-x (SCP-x) were significantly upregulated at these stages. These findings demonstrate for the first time that ACBP is an essential protein required for embryonic development and its loss of function may be initially compensated by concomitant upregulation of two other LCFA-CoA binding proteins, but only at the earliest pre-implantation stages. The fact that ACBP is the first known intracellular lipid binding protein whose deletion results in embryonic lethality suggests its vital importance in mammals.

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Keywords

ACBP; DBI; Gene targeting; Pre-implantation embryonic lethality

Introduction

Acyl-CoA binding protein (ACBP), also known as diazepam-binding inhibitor (DBI), is a soluble 10-kDa lipid-binding protein ubiquitously expressed in all tissues of eukaryotic species examined [1,2]. ACBP expression differs significantly among cell types and is highly regulated by hormones (insulin, androgens). Via the ACBP promoter, ACBP expression is also determined by several nuclear transcription factors important in lipid and glucose metabolism: peroxisome proliferator-activated receptors (PPARs) α and γ as well as sterol regulatory element binding protein (SREBP) [3–7]. Unique among the intracellular lipid protein families, ACBP exhibits very high affinity (<10 nM K_d s) and specificity exclusively for long-chain fatty acyl-CoAs (LCFA-CoAs) [8,9]. LCFA-CoAs are potent regulators of a wide variety of enzymes, signaling receptors, and nuclear regulatory proteins involved in fatty acid and glucose metabolism [1,10,11]. In vivo, pancreatic insulin secretion is affected by LCFA-CoA and ACBP levels as well as by glucose [12–14]. Most important, the key enzymes in de novo fatty acid synthesis (ACAC, acetyl CoA carboxylase; FASN, fatty acid synthase) are inhibited by the LCFA-CoA end product ($K_i < 50$ nM) [10,15]. By binding LCFA-CoA, ACBP removes this end-product inhibition to stimulate ACAC and FAS [10,15]. Likewise, by binding and reducing the unbound levels of LCFA-CoAs, ACBP plays important roles not only in normal regulation of LCFA-CoA transport, metabolism, signaling, vesicular trafficking, and nuclear regulation, but also in opposing the deleterious effects of elevated intracellular LCFA-CoA levels associated with diabetes and obesity [10,13]. The physiological relevance of ACBP is supported mainly by studies of ACBP overexpression in yeast, mice, rats, and plants (*Arabidopsis*) [16–18]. ACBP-overexpressing mice fed control chow exhibit altered hepatic lipid metabolism [9]. ACBP-overexpressing rats fed a medium-chain fatty acid-rich diet have improved glucose tolerance and lower serum insulin levels [18]. Finally, a single nucleotide polymorphism in the human ACBP gene promoter is associated with reduced risk of type 2 diabetes in two German study populations—probably due to increased transcriptional activity of ACBP [19].

While the above findings suggest that loss of ACBP could result in major disruptions of normal phenotype and possible lethality, the available evidence to date is unclear. Depletion of the ACBP protein in the wild-type DTY10A yeast strain results in a slower growing phenotype, which subsequently adapts to a faster growing phenotype (frequency $> 1:10^5$), while other ACBP-null yeast strains rapidly adapt, such that growth rate is unaffected [20,21]. A conditional ACBP knock-down in yeast alters lipids, membranes, and vesicle accumulation, but is not lethal [20]. Although disruption of the 10-kDa ACBP gene in the plant *Arabidopsis* is not lethal, *Arabidopsis* expresses at least five additional ACBP genes that also bind LCFA-CoAs—probably compensating for the loss of the 10-kDa ACBP [22,23]. Multiple independent genes encode different functional paralogues of ACBP even within a single species [2,24]. In mammals, these include: (1) 10-kDa ACBP [also called liver ACBP (L-ACBP)] and its two distinct homologues (testes, T-ACBP; brain, B-ACBP); (2) ACBP-like domains in several large, multifunctional proteins; and (3) multiple inactive pseudogenes [2,24]. Several studies with transformed mouse and human cell lines suggest that knock-down of ACBP (ACBP antisense RNA or siRNA) is very deleterious—inhibiting differentiation or resulting in lethality [25,26]. However, because transformed cells are often deficient in the other LCFA-CoA-binding proteins [1,11,27,28], it is difficult on this basis alone to predict if ACBP is an essential protein in mammals. While recent studies with mice carrying the spontaneous nm1054 mutation suggest that deletion of ACBP is not an

embryonic lethality, this conclusion is complicated by the nature of the nm1054 mutation, which arose in CBA/J mice as a result of a large genomic deletion (about 400 kb) that contains all or part of at least six genes, only one of which encodes ACBP [29–31]. On a homozygous C57BL/6J mouse background, the nm1054 mutation results in significant prenatal lethality, and the very few live-born homozygotes almost all die before weaning [31]. However, on a mixed background comprised of at least two different mouse strains, the nm1054 mutation is not embryonically lethal, but instead the mice live to adulthood and exhibit a phenotype characterized by sparse hair, skin lipid metabolic abnormalities, male infertility (uniformly infertile on all genetic backgrounds), failure to thrive, hydrocephaly, and anemia [29–31]. Consequently, the complexity of the nm1054 mutation makes it difficult to assign the individual contribution(s) of ACBP to the phenotype independent of the other five concomitantly deleted genes, and/or down-stream effects of deleting the promoter and intronic regions of all six genes.

The purpose of the present investigation was to resolve whether ACBP is an essential protein in a mammalian system by ablating only the ACBP gene function by homologous recombination in mice. The data show that loss of ACBP resulted in early pre-implantation embryonic lethality by the 8-cell stage. The fact that ACBP is the first known intracellular lipid binding protein whose single gene deletion results in embryonic lethality suggests its vital importance in mammals.

Materials and Methods

Materials

Construct Preparation—A BAC clone containing the known expressed ACBP sequence was obtained from the BACPAC Resource Center (Oakland, CA). This clone was fully sequenced to confirm the presence of the full-length expressed mouse ACBP gene rather than one of many inactive mouse pseudogenes (DNA Technologies Core Laboratory, Texas A&M University). Restriction enzymes were from Invitrogen (Carlsbad, CA) while DNA purification kits (Miniprep, Maxiprep, Agarose Gel Extraction kits, PCR purification kits) were from Qiagen (Valencia, CA). Prime-A-Gene labeling was from Promega (Madison, WI), and oligonucleotides from Integrated DNA Technologies (Coralville, IA)

Embryonic Stem Cells—The 129S6-derived embryonic stem (ES) cell line W4 was from Taconic Inc. (Hudson, NY) while primary mouse embryonic fibroblasts (PMEF) were from Specialty Media (Phillipsburg, NJ). Fetal bovine serum was from Summit Biotechnology (Fort Collins, CO) while cell culture media and components (non-essential amino acids, penicillin, streptomycin, L-glutamine, G418, sodium pyruvate) were from Invitrogen (Carlsbad, CA). Leukemia inhibitory factor (LIF, ESGRO[®]) was from Chemicon (Temecula, CA).

Embryo Isolations—M2 and M16 media, Pregnant Mare Serum Gonadotropin (PMSG), human chorionic gonadotropin (hCG), mineral oil and hyaluronidase were from Sigma-Aldrich (St. Louis, MO), Potassium Simplex Optimized Medium (KSOM) was from Millipore (Billerica, MA), and ES cell injection needles and blastocyst holding capillaries were from Eppendorf (Hamburg, Germany).

Animals—All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. Male and female inbred C57BL/6NCr mice were from the National Cancer Institute (Frederick Cancer Research and Developmental Center, Maryland). ACBP genetically-engineered mice were generated as described in the section below. All mice were maintained in microisolators, with a 12 h light/dark cycle in a

temperature-controlled facility (25 °C), access to standard commercial rodent chow (Teklad®, Harlan, Indianapolis, IN, USA) and water ad libitum. Mice were monitored by the presence of sentinels quarterly and found negative for all known mouse pathogens.

Generation of ACBP Gene-Ablated Mice—The ACBP-null targeting construct was designed to replace the N-terminal promoter region (including the known SREBP and PPAR γ response elements), exon 1, intron 1, exon 2, and part of intron 2 of the ACBP gene with a neomycin cassette. The following two overlapping genomic DNA fragments from mouse clone RP23-430P22 (BACPAC Resources Center, BPRC, Oakland, CA, USA) were used to form the backbone of the ACBP gene targeting construct: a 7 kbp *XhoI* clone containing the promoter region, exon 1, exon 2, and the surrounding intronic sequences of the ACBP gene, and a 7 kbp *HindIII* clone containing exons 1 through 3 and surrounding intronic sequences. ACBP genomic sequences were confirmed by extensive restriction mapping and DNA sequencing (DNA Technologies Core Laboratory, Texas A&M University). The 5' arm of homology was generated by ligating a 4.2 kbp *XhoI/SmaI* fragment from the 7 kbp *XhoI* clone into pBlueScript-SK (pBS-SK; Stratagene, La Jolla, CA, USA). An intermediate targeting construct consisting of the neomycin resistance marker and a 3.9 kbp *XhoI/HindIII* fragment from the 7 kbp *HindIII* clone was generated by ligating the neo cassette from a pPGK-Neo vector to the 3.9 kbp *XhoI/HindIII* fragment. Ligating the 5' homology arm with the intermediate targeting construct pre-digested with *SmaI* completed the targeting construct. Once complete, the targeting construct was linearized with *NotI* and electroporated into the W4 ES cell line maintained on a PMEF feeder layer. Disruption of the ACBP gene was generated through homologous recombination. After selection with G418, DNA was isolated from surviving clones, digested with *HindIII*, and screened by Southern blotting analysis following standard protocols. Using a 760-bp 3' probe, targeted clones were identified by Southern blotting with the presence of a 4.5-kbp band while absence of the targeting construct was indicated by a 7-kbp band. Southern blotting used a 3' probe constructed from sequence immediately after the disrupted locus (between *Bam/Sma* and *HindIII*). Four positive clones were expanded and injected into C57BL/6Ncr blastocysts to create chimeric mice following standard procedures. Four male chimeras from two separate ES cell clones were identified by coat color and bred to C57BL/6Ncr females to determine germ-line transmission of the targeted allele. Tail DNA from the chimera/wild-type backcross F1 off-spring were initially screened with Southern blotting by standard procedures to verify germline transmission. Subsequent generations of heterozygote/heterozygote and wild-type crosses (for PCR controls) were genotyped by PCR, with the following primer sets: forward primer (ACBP-anchor, 5'-CAA CCT CTG CCA TCA CCT ATT C-3'); reverse primer wild type (ACBP-wt, 5'-TTC TCT GTA TAG CTC TGG CTG G-3') and reverse primer gene ablation (ACBP-ko, 5'-GGT GGC TAC CCG TGA TAT TG-3'), for 35 cycles with an annealing temperature set at 58 °C. The ACBP-null mice described herein were backcrossed to C57BL/6Ncr mice for at least six generations.

Isolation of Pre- and Post-Implantation Stage Embryos—ACBP heterozygous or wild-type (control) females were paired overnight with ACBP heterozygous or wild-type males, respectively and checked for the presence of a copulation plug the next morning. The day of the copulation plug was designated 0.5 post coitum (dpc). Females were humanely euthanized by cervical dislocation immediately prior to embryo isolation. Post-implantation embryos were obtained by dissecting the uterus at 9.5, 11.5, 14.5 and 17.5 dpc. These embryos were freed of any extra-embryonic tissue and then prepared for PCR analysis. Pre-implantation embryos were obtained by flushing the oviducts or uterus, depending upon the time point. One-cell and two-cell (cleavage) oocytes were isolated from the oviducts the same morning of the copulation plug (0.5 dpc) and the following morning (1.5 dpc),

respectively. Eight-cell (morula) stage embryos were isolated from the oviduct on the third day (2.5 dpc) and blastocysts were obtained by flushing the uterus on the fourth day (3.5 dpc). Hepes-buffered M2 medium was used to flush and handle all embryos, and KSOM under mineral oil was used for up to 6 days for in vitro culture of all pre-implantation embryos at 37 °C, 5% CO₂. One-cell stage embryos were treated with 1 mg/ml of hyaluronidase and subsequently rinsed 5–10 times in sterile M2 medium to remove cumulus cells. The development and morphology of pre-implantation stages were monitored by visualizing the embryos with an inverted phase contrast microscope (Nikon Diaphoto 300, Nikon, Tokyo, Japan) at 12-h intervals after oocyte collection.

Genotyping of Pre- and Post-Implantation Embryos—Genotyping of individual pre-implantation embryos was performed using the REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Genomic DNA from post-implantation embryos was obtained by digesting a small section of tail in 500 µl of lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 300 mM Na acetate, 1% SDS, 0.2 mg/ml proteinase K) for 6 h at 55 °C, followed by 20 min at 95 °C to inactivate the proteinase K. The lysate was used directly for PCR genotyping. The same PCR primer sets to genotype embryos were used as with the live offspring. Initial embryo genotyping was performed on embryos isolated from the F2 intercross heterozygote generation. All embryos genotyped for real-time reverse transcriptase polymerase chain reaction (Q-rtPCR) were from the N6 heterozygote intercross generation or greater.

Quantitative (Real-Time) Reverse Transcriptase PCR—Q-rtPCR was performed on total RNA from 2.5-dpc embryos isolated and purified using RNeasy Micro kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. Expression patterns were analyzed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using TaqMan[®] One Step PCR Master Mix Reagent kit, gene specific TaqMan[®] PCR probes and primers, and the following thermal cycler protocol: 48 °C for 30 min for reverse transcription prior to amplification, 95 °C for 10 min before the first cycle, 95 °C for 15 s, and 60 °C for 1 min, repeated 60 times. For other specific probes and primers, TaqMan[®] Gene Expression Assay products for mouse peroxisome proliferator-activated receptor- α (Ppara, Mm00440939_m1); sterol carrier protein 2 (Scp-2, Mm01257982_m1); m-calpain (Capn2, Mm00486669_m1), and Custom-TaqMan[®]-Assay products sterol carrier protein-x (Scp-x, SEQ_SCPX-EX23); acyl-CoA binding protein (Acbp, SEQ_ACBP). Measurements were performed in duplicate and analyzed with ABI PRISM 7000 SDS software to determine the threshold cycle (C_T) from each well. Primer concentrations and cycle number were optimized to ensure that reactions were analyzed in the linear phase of amplification. To analyze the Q-rtPCR data, mRNA expression of Ppara, Scp-2, Scp-x, and Capn2 in homozygous null pre-implantation embryos were normalized to a housekeeping gene (18S rRNA); made relative to the control wild-type pre-implantation embryos, and calculated using the comparative $2^{-\Delta\Delta C_T}$ method [32], where $\Delta\Delta C_T = [C_T \text{ of target gene} - C_T \text{ of } 18 \text{ s}]_{\text{ACBP-null embryos}} - [C_T \text{ of target gene} - C_T \text{ of } 18 \text{ s}]_{\text{wild type embryos}}$ as described in User Bulletin 2, ABI PRISM 7000 SDS.

Results

Generation of ACBP Gene-Ablated Mice

The strategy for generation of a mouse ACBP-null targeting construct was dictated by the biological activity of ACBP proteolytic fragments as well as the unique nature of the ACBP gene itself. For example, the N-terminal region of ACBP is the precursor of two major biologically active peptides active in lipid metabolism, signaling, and insulin secretion [14,33,34]. In addition, alternative splicing in the mouse ACBP gene results in transcription

of two mRNA transcripts encoding proteins of 86 and 135 amino acids, respectively [7]. Although western blotting of mouse liver homogenates with several different polyclonal anti-ACBP antisera detected the 10-kDa ACBP coded by the transcript for 86 amino acids, these antisera did not detect a 15.9-kDa ACBP coded by the alternate transcript for 135 amino acids (not shown). Nevertheless, the mouse ACBP-null targeting construct was designed to replace the N-terminal promoter region (including the known SREBP and PPAR γ response elements), exon 1, intron 1, exon 2, and part of intron 2 of the ACBP gene with a neomycin cassette (Fig. 1a). Chimeric ACBP gene ablated mice were developed using this construct as described in Methods. The offspring from chimera/wild-type backcrosses were genotyped by Southern blotting using a 3' probe constructed from sequence almost completely after the disrupted locus (horizontal solid bar after exon 3 and *Bam/Sma*). Southern blotting of *HindIII*-digested DNA revealed wild-type (7 kB) and targeted heterozygous (4.5 kB) DNA (Fig. 1b). A PCR screen from tail clips was then developed (Fig. 1c), using the two primer sets as described in Methods, for genotyping all other offspring.

ACBP Heterozygous Mice Are Phenotypically Normal

Wild-type and heterozygous mice were indistinguishable with respect to visual appearance, post-implantation embryonic weight (Fig. 2a), body weight at weaning (not shown), adult body weight (Fig. 2b), and fertility (not shown). Since only one of the two ACBP alleles was deleted in the heterozygous mice, rtPCR and Western blotting detected only the expected 10-kDa ACBP in livers of both wild-type and heterozygous mice (not shown).

The ACBP-Null Mutation is Embryonically Lethal

The F1 ACBP heterozygotes were intercrossed to produce ACBP-null offspring. Instead of the expected Mendelian 1:2:1 ratio of 25% wild-type, 50% heterozygous, and 25% null ACBP mice, examination of 171 total F2 offspring from 21 litters yielded no homozygous null ACBP pups (Fig. 3). The remaining greater than 2:1 ratio of heterozygous to wild-type mice was consistent with the absence of adverse effects of the mutant allele in the heterozygotes (Fig. 3), and average litter size was within the normal range of a hybrid B6:129 strain (D. Landrock, unpublished data). Pregnant females were carefully monitored until the day of parturition. No evidence for increased neonatal lethality or cannibalization was noted. Taken together, these data indicated a potential embryonic lethality.

Genotypes of Post-implantation Embryos

To determine if ACBP gene ablation resulted from a post-implantation embryonic lethality, the genotypes of post-implantation F2 embryos from F1 ACBP heterozygote/heterozygote intercrosses were determined. Of the 35 post-implantation embryos isolated, no ACBP homozygous null embryos were recovered 9.5 dpc (E15, 9 total, Fig. 4a), 11.5 dpc (E19, 8 total, Fig. 4b), 14.5 dpc (E22, 9 total, Fig. 4c), and 17.5 dpc (E25, 9 total embryos, Fig. 4d). No evident indicators of resorbed embryos were found during dissection. Thus, ACBP-null embryos never formed, or died prior to or very shortly after implantation.

Genotypes of Pre-implantation Embryos

To determine if ACBP-null mice resulted in a pre-implantation embryonic lethality, the genotypes of 310 of 510 pre-implantation F2 embryos from F1 ACBP heterozygote intercrosses were determined at the 2-cell (oocyte), ~8-cell (morula), and ~32-cell (blastocyst) stages. At the oocyte stage, the amount of DNA recovered for PCR was minimal; thus it was not possible to identify the genotype of all oocytes at this stage. Of over 200 oocytes recovered, 36 were clearly identified as to genotype, of which 4 were the homozygous null ACBP genotype (Table 1). As shown by representative light microscopic

images, the null ACBP oocytes (Fig. 5a) did not differ in appearance from wild-type oocytes (Fig. 5e). Of 146 morulae recovered, 17 were the ACBP homozygous null genotype (Table 1). Similarly, light microscopy showed that the ACBP-null morulae (Fig. 5b) did not differ significantly in appearance from wild-type (Fig. 5f). In contrast, while 128 blastocysts were recovered and genotyped, none were the ACBP-null genotype (Table 1). Furthermore, light microscopy showed that some blastocysts appeared to be undergoing degeneration (Fig. 5d), while the majority appeared normal (Fig. 5h). Due to DNA degradation, it was not possible to definitively genotype the degenerating blastocysts. These dead/dying blastocysts most probably represented ACBP homozygous null embryos. Thus, ACBP gene ablation resulted in early pre-implantation embryonic lethality beginning by the 2.5-dpc morula (8-cell) stage, and none were viable by the 3.5-dpc blastocyst (~32-cell) stage.

Q-rtPCR of ACBP in Pre-implantation Embryos

To determine if the lethality was associated with total absence of ACBP transcription, 2.5 and 3.0-dpc embryos from ACBP heterozygote intercrosses were examined by Q-rtPCR. The ACBP transcript was detected in wild-type and heterozygous embryos at the 2.5-dpc morula (Fig. 6a, b) and 3.0-dpc stage (Fig. 6b) just prior to blastocyst formation. In contrast, no *Acbp* gene transcript or alternate spliced longer transcript was detected in the morula 2.5-dpc stage of ACBP-null embryos (Fig. 6a, b). The absence of *Acbp* transcript in the 2.5-dpc null embryos was consistent with correct insertion of the construct into the *Acbp* gene. Detection of *Acbp* transcript in 3-dpc ACBP-null embryos was not possible because no ACBP-null embryos were found by this late morula/early blastocyst stage (Fig. 6b, ∞). Thus, the *Acbp* transcript was absent in the ACBP homozygous null 2.5-dpc stage morula, preceding the embryonic lethality by the blastocyst 3–3.5-dpc stage (Figs. 5d, 6b). Western blotting to determine presence of the ACBP protein in the null morulae was not possible due to the limited amount of material present at this early stage of development.

Concomitant Upregulation Of Other Long-Chain Fatty Acyl-CoA Binding Proteins

Since ACBP gene ablation was not lethal at the morula 2.5-dpc stage, the possibility that loss of ACBP was temporarily compensated for at least in part by upregulation of another cytosolic LCFA-CoA binding protein was examined. Sterol carrier protein-2 (SCP-2) and sterol carrier protein-x (SCP-x) bind LCFA-CoAs with similar affinities as ACBP [8,35]; their ablation is not lethal [36–38]; and they are expressed as early as the zygote 1-cell stage, slightly earlier than ACBP at the 2-cell stage (Supplemental Fig. S1A). *Scp-2* expression in ACBP-null 2.5-dpc morulae was upregulated tenfold (Fig. 6a). Likewise, expression of *Scp-x* in ACBP-null morulas (2.5 dpc) was upregulated over 50-fold (Fig. 6a). These data suggest that significant upregulation of SCP-2 and SCP-x may indicate an attempt to compensate for the loss of ACBP to maintain viability.

The possibility that ACBP gene ablation may result in compensatory upregulation of the nuclear peroxisome proliferator-activated receptor- α (PPAR α) was also investigated. PPAR α exhibits high affinity for LCFA-CoAs [39,40] and PPAR α coactivator recruitment and transcriptional activity are regulated by both LCFA-CoAs and ACBP [39–42]. Furthermore, expression of the PPAR α transcript occurred by the zygote (1-cell) stage, thereby preceding the appearance of ACBP, which normally occurs at the oocyte cleavage (2-cell) stage (Supplemental Fig. S1A). However, ACBP gene ablation did not significantly alter the expression of *Ppar α* transcript at the morula (2.5-dpc) stage. Thus, viability of very early pre-implantation ACBP-null embryos was not associated with concomitant upregulation of PPAR α .

Since ACBP is a potent activator of m-calpain, a protease involved in apoptosis [43], the possibility that m-calpain transcript (*Capn2*) is expressed at an early pre-implantation

embryonic stage was investigated. *Capn2* was not expressed in the morula (2.5 dpc) embryonic stage of ACBP-null, heterozygote, or wild-type embryos (Fig. 6b). Since *Capn2* was not detected until 3.0 dpc and no ACBP-null pre-implantation embryos were detected by that stage, embryonic lethality at >2.5 dpc was not associated with ACBP activation of m-calpain.

Effect on Unbound Free Long-Chain Fatty Acyl-CoA (LCFA-CoA) Concentration

LCFA-CoAs are potent regulators (K_i s as low as 50 nM) of many enzymes, transporters, and receptors involved in lipid and glucose metabolism (Supplemental Table S1). Since determination of LCFA-CoA and ACBP protein concentrations would require more material than is present in these early stage embryos to be detectable, the effect of ACBP on the free unbound LCFA-CoA concentration was modeled over the known physiological range of LCFA-CoAs and ACBP concentrations in mammalian tissues (see Supplementary Materials, Methods). At the upper range of physiological ACBP concentration (50 μ M), ACBP very effectively buffered the unbound LCFA-CoA concentrations over a relatively broad range (Supplemental Fig. S2A). The unbound LCFA-CoA concentration was <50 nM up to 40 μ M total LCFA-CoA (Supplemental Fig. S2B). In contrast, estimated lower physiological ACBP concentration (6 μ M) was relatively ineffective in buffering the unbound LCFA-CoA level (Supplemental Fig. S2A), which was >50 nM even at 10 μ M total LCFA-CoA (Supplemental Fig. S2B). Taken together, these data suggest that the loss of ACBP would probably result in significantly increased free unbound LCFA-CoA levels within the null embryo cells, which in turn would adversely affect many proteins, enzymes, and receptors involved in both lipid and glucose metabolism.

Discussion

Although long-chain fatty acyl CoAs (LCFA-CoAs) are well known intermediates in fatty acid metabolism, they are also potent metabolic regulators of multiple enzymes/proteins involved in fatty acid and glucose metabolism [1,10,13]. While physiologic tissue total LCFA-CoA levels are in the 5–150 μ M range [1,10,13], even surprisingly low levels (50–500 nM) of free unbound LCFA-CoAs inhibit a broad variety of enzymes, transporters, signaling receptors, and nuclear receptors involved in key cellular processes such as fatty acid and cholesterol synthesis, transcription of genes in lipid metabolism, mitochondrial fatty acid oxidation, and glucose metabolism (Supplemental Table S1). The respective K_i s by LCFA-CoA for many of these proteins are as much as 3,000-fold lower than the range of total LCFA-CoA levels in tissues [1,10,13]. Since these proteins are known to be active in tissues, the actual unbound free LCFA-CoA levels are much lower (10–20 nM) due to buffering by intracellular LCFA-CoA binding proteins—especially by ACBP (rev. in [1,10,13]. The present investigation determining the effect of ACBP gene ablation on the phenotype of mice yielded the following insights.

First, the phenotype of ACBP gene-ablated mice on a C57BL/6NCr background was more severe than that of the more complex nm1054 spontaneous mouse mutation on a C57BL/6J or mixed strain background. Loss of only the ACBP gene function was embryonically lethal at the pre-implantation stage in ACBP-null mice on a C57BL/6NCr background. While spontaneous loss of ACBP together with at least five other genes (an earlier study indicated as many as 200 genes) also resulted in significant prenatal lethality in homozygous nm1054 null mice on a C57BL/6J background, almost all of the few live-born pups die before weaning and few have been weaned successfully [30,31]. In contrast, loss of ACBP (along with 5 other genes) elicited a somewhat milder phenotype (sparse hair, skin lipid metabolic abnormalities, male infertility, failure to thrive, hydrocephaly, anemia) in homozygous nm1054 mice on a mixed (two different strains) mouse background [29–31]. It is yet to be determined why the nm1054 mutation resulting in loss of ACBP as well as five other genes

is less ‘lethal’ than deletion of the ACBP gene alone. While it cannot be ruled out that loss of the promoter sequences in our construct may have also affected the expression of other genes and thereby contributed to pre-implantation embryonic lethality, it is unclear why the loss of even more promoter sequences due to deletion of six genes in the nm1054 mutation resulted in a less severe phenotype. One possibility is that the loss of one or more of the five additional genes (i.e. loss of exons as well as intronic sequences, and N-terminal promoter sequences) may have contributed in an as yet not understood manner to compensate for the loss of the ACBP. The different mouse strain backgrounds of the ACBP-null mice (C57BL/6NCr) and nm1054 mice (C57BL/6J or mixed CBA, 129) may also have played a role, since phenotype versus strain differences have also been noted in ACBP gene deletions in different yeast strains [20,21] as well as deletions of other gene-ablated mouse models [44,45]. The importance of genetic background is emphasized by a recent report comparing two yeast strains (Σ 1278b and S288c) wherein about 5,100 genes were systematically deleted [46]. While 894 genes were essential in both strains, 44 genes were essential only in Σ 1278b and 13 genes were essential only in S288c. Examination of hybrid strain crosses of 18 mutants that were lethal in Σ 1278b, but not in S288c, yielded viable progeny in all cases and their conditional phenotype was associated with numerous modifier genes that differ between the strains [46]. On the basis of this and previous work, it was concluded that “conditional essentiality is almost always a consequence of complex genetic interactions involving multiple modifiers associated with strain specific genetic variation rather than classical digenic synthetic lethality” [46]. As has been seen in many other cases, genetic background can profoundly affect the phenotypes manifest upon gene ablation. While such ‘discrepancies’ often cause controversy, they also identify potentially important areas of future research.

Second, deletion of the 10-kDa ACBP encoding gene in the mouse is the first known embryonically lethal mutation in any of the intracellular LCFA-CoA binding protein families. At least three families of soluble lipid binding proteins appear to buffer cytoplasmic LCFA-CoA levels by binding LCFA-CoAs with high affinity, in the order: acyl-CoA binding protein (ACBP) [1,10,13] >sterol carrier proteins (SCP-2, SCP-x) [47] >select fatty acid binding proteins [FABP1 (L-FABP) >>FABP7, FABP2, FABP3 (B-FABP, I-FABP, H-FABP)] [48–50]. Of these LCFA-CoA binding proteins, only ACBP, SCP-2/SCP-x, and FABP3 are expressed in pre-implantation embryos (Supplementary Figure S1). However, FABP3 (H-FABP) binds LCFA-CoA even more weakly than FABP1 (L-FABP) — a protein not expressed in pre-implantation embryos (Supplementary Figure S1) [48,49]. Thus, redundancy in LCFA-CoA binding proteins is much more limited in pre-implantation embryos than in more mature tissues. These data would suggest that loss of one of the lower affinity LCFA-CoA binding proteins would not probably be lethal, and to date lethality has not been observed upon gene ablation of several members of the large FABP protein family, including: Fabp1 (liver L-FABP), Fabp2 (intestinal I-FABP), Fabp3 (heart H-FABP), Fabp4 (adipocyte A-FABP), Fabp5 (keratinocyte K-FABP), and Fabp7 (brain B-FABP) [44,51,52]. Likewise, ablation of SCP-2 and SCP-x is not lethal [37,38,53]. Even loss of both L-FABP and SCP-2/SCPx (intercross of L-FABP null mice and SCP-2/SCP-x null mice) does not result in lethality [54]. Thus, embryonic lethality in mice ablating the gene encoding the 10-kDa ACBP is unique among the multiple LCFA-CoA binding proteins known to exist in the cytoplasm of mammalian tissues.

Third, the ACBP gene ablation-induced embryonic lethality at very early pre-implantation stages where the embryo is not well differentiated was consistent with studies showing that ACBP-specific siRNA treatment of highly undifferentiated mouse and human tumor cells is lethal [26]. The observed lethality of ACBP-specific siRNA treatment was probably due to loss of LCFA-CoA buffering capacity concomitant with loss of ACBP in the context of the already markedly reduced levels of other LCFA-CoA binding proteins (e.g. FABPs, SCP-2/

SCPx) in transformed cells (rev. in [1,11,27,28]. The fact that some apparently normal ACBP-null embryos, albeit reduced in expected numbers, were still alive at the 1–8 cell pre-implantation stages, but not at later embryonic stages, was most probably due to the presence of residual maternal ACBP protein. Consistent with the fact that almost all maternal mRNA is degraded by the end of the 2-cell stage [55,56], the ACBP mRNA was not longer detected in the 8-cell stage pre-implantation embryos. However, since the ACBP protein has a relatively long half life (i.e. $t_{1/2}$ of 25–53 h) [55–57], some maternal ACBP protein was probably present in the 4-cell, but not in the 8-cell pre-implantation stage. Concomitant upregulation of two less prevalent LCFA-CoA binding proteins (SCP-2 and SCP-x, primarily in peroxisomes) shown herein may have also facilitated survival at the morula (8 cell) stage, but was insufficient to assure survival by the blastocyst (~32 cell, 3.5-dpc) stage.

Fourth, the early pre-implantation embryonic lethality in ACBP-null mice was consistent with the importance of ACBP in preventing deleterious effects of LCFA-CoAs on sensitive enzymes involved in energy production and fatty acid biosynthesis. While fatty acids (primarily derived from endogenous triglycerides) are the major energy source in unfertilized oocytes, thereafter very little energy is derived from fatty acids and intracellular triglyceride is maintained relatively constant [58]. Since there is too little glycogen to sustain the pre-implantation embryo, especially that of the mouse, the pre-implantation embryo is heavily dependent on an exogenous energy supply for synthesizing fatty acids [58]. Pyruvate and lactic acid in oviductal fluid represent the major carbon sources of mouse pre-implantation embryos up to the 4-cell stage, while glucose serves this purpose thereafter [58–60]. Acetyl CoA derived from these nutrients is oxidized to produce energy or is used as a substrate for fatty acid synthesis required for formation of membranes and longer-term energy storage than can be accomplished by energy storage as glycogen. The two key enzymes in fatty acid synthesis [acetyl CoA carboxylase (ACAC, rate limiting); fatty acid synthase (FASN)] are both end-product inhibited by fatty acyl-CoA [10,15]. Acetyl CoA carboxylase is especially sensitive (K_i 50 nM) to the presence of even very low levels of LCFA-CoA [10]. By binding LCFA-CoAs, ACBP very effectively removes the LCFA-CoA end-product inhibition of ACC and FASN [10,15]. The importance of these fatty acid synthetic enzymes in early embryogenesis is underscored by the fact that ablation of ACC or FASN results in an early embryonic and pre-implantation embryonic lethality [61,62]. As shown herein, loss of ACBP (the major high-affinity LCFA-CoA binding protein) is expected to eliminate most of the LCFA-CoA buffering capacity. The other major LCFA-CoA binding protein (FABP1, i.e. L-FABP) is not induced until much later in development. While two other LCFA-CoA binding proteins (SCP-2, SCP-x) are present and concomitantly upregulated in ACBP-null pre-implantation embryos, these proteins are largely compartmentalized in peroxisomes and are present at much lower level (rev. in [47]). Since ACBP is thought to be the most effective of the LCFA-CoA binding proteins in buffering the unbound free LCFA-CoA concentration [1,10,63], complete loss of ACBP would thus be expected to greatly increase unbound free LCFA-CoAs over that normally present in cells and tissues—thereby inhibiting ACC and FASN enzymes essential for de novo fatty acid synthesis.

Fifth, ACBP exhibits a unique role in early pre-implantation embryonic development as compared to the other intracellular LCFA-CoA binding proteins. Several other cytoplasmic LCFA-CoA binding proteins (FABPs 5, 4, and 3; SCP-2, SCP-x) are expressed already by the zygote 1-cell to morula 8-cell stages (Supplemental Fig. S1), but gene ablation of many of these proteins is not lethal [37, 38, 44, 51–53]. ACBP is also known to enter the nucleus, bind nuclear receptors (PPAR α , PPAR γ) involved in lipid and glucose metabolism, and regulate transcriptional activity of these receptors [11, 64–66]. PPAR α itself exhibits high affinity for and is regulated by LCFA-CoAs transported by ACBP [11, 39, 40]. While

PPARs α and γ are both present at the zygote one-cell stage (Supplemental Fig. S1), ablation of PPAR α is not lethal, and ablation of PPAR γ is lethal but not until much later, i.e. the post-implantation E10 stage [67, 68]. Thus, it is unlikely that dysregulation of PPARs in response to ACBP deletion accounts for the observed very early pre-implantation lethality.

Finally, the 10-kDa ACBP does not appear to be an essential protein in other eukaryotes, since its deletion results in the appearance of revertants (yeast) and probably compensated by distinct other ACBP genes in the nematode (*C. elegans*), insect (*D. melanogaster*), and plants (*Arabidopsis*) [20,22,23,69–71]. It is also important to note that in mammals the gene encoding the 10-kDa ACBP also expresses two additional transcripts (due to alternate transcription initiation) encoding distinct ACBP homologues (testes T-ACBP; brain B-ACBP) [2,24]. In silico analyses of several databases (NCBI UniGene, <http://www.ncbi.nlm.nih.gov/unigene>; European Bioinformatics Institute, <http://www.ebi.ac.uk>) indicates the presence of expressed sequence tags (ESTs) for all three mRNAs derived from the human ACBP gene (only two have active promoters), for only the mRNA encoding the rat 10-kDa ACBP, and for two mRNAs encoding the mouse 10-kDa ACBP (87 amino acid) and a mouse 15.9 kDa (135 amino acid) ACBP-related protein [7]. While two additional mouse ESTs [one from brain (BE652768) and one from testis (AA4927130)] have been reported [7], a search of several databases attributed both of these ESTs to the known gene encoding the 10-kDa ACBP protein (Mouse Genome Informatics Database, Jackson Labs, Bar Harbor, ME; NCBI UniGene database, October 2009, <http://www.ncbi.nlm.nih.gov/unigene>). Mammals (mouse, rat, human) also process multiple inactive pseudogenes [NCBI UniGene database, October 2009, <http://www.ncbi.nlm.nih.gov/unigene>]; [2,24]]. While the physiological relevance of potentially two mRNA transcripts of the mouse ACBP gene in early pre-implantation embryonic development is not clear, the mRNA encoding ACBP was present in all wild-type mouse embryos examined but not in homozygous ACBP-null 8-cell stage pre-implantation embryos or thereafter.

In summary, the studies presented herein addressed for the first time the role of ACBP single gene ablation on development in mice. ACBP gene ablation resulted in pre-implantation embryonic lethality between the morula (8 cell) and blastocyst (32 cell) stages. In contrast to ACBP-null mouse, gene ablation of several other cytosolic LCFA-CoA binding proteins also expressed at these early pre-implantation embryonic stages is not lethal. Thus, ACBP represents the first discovered LCFA-CoA binding protein whose ablation results in lethality. While the exact role of ACBP in normal pre-implantation embryonic development remains to be identified, at least two general possibilities may be considered: (1) Since the high affinity of ACBP for LCFA-CoAs results in highly effective buffering of total LCFA-CoAs to maintain unbound free LCFA-CoAs at low levels, loss of ACBP probably results in a significant increase of unbound free LCFA-CoA levels. The elevated unbound free LCFA-CoA levels would consequently inhibit highly LCFA-CoA sensitive enzymes (e.g. acetyl CoA carboxylase, fatty acid synthase) required for normal glucose metabolism and fatty acid synthesis in these rapidly growing pre-implantation embryos; and (2) Since ACBP normally interacts with and regulates several nuclear receptors (e.g. PPARs) present in early pre-implantation embryos, loss of such interactions may result in abnormal transcriptional regulation of genes involved not only in lipid and glucose metabolism but also development. Both factors could contribute to the lethality observed in these early pre-implantation ACBP-null embryos. Future studies using an inducible construct or a conditional knockout approach (e.g. as applied to acetyl-CoA carboxylase and fatty acid synthase [72,73]) should allow further discrimination of the physiological functions of ACBP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACAC	Acetyl CoA carboxylase
ACBP	Acyl-CoA binding protein
CAPN2	m-calpain
CoA	Coenzyme A
DBI	Diazepam binding inhibitor protein
FASN	Fatty acid synthase
L-FABP	Liver fatty acid binding protein
LCFA-CoA	Long-chain fatty acyl-CoA
PCR	Polymerase chain reaction
PPARα	Peroxisome proliferator-activated receptor- α
PPARγ	Peroxisome proliferator-activated receptor- γ
SCP-2	Sterol carrier protein-2
SCP-x	Sterol carrier protein-x
SREBP	Sterol regulatory element binding protein

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Fig. 1. Construct design and screening of ACBP gene-ablated mice. **a** The targeting construct, wild-type ACBP locus and targeted locus after homologous recombination. Two lower diagrams show the wild-type locus and targeted ACBP locus with expected *HindIII* fragment sizes with the indicated probe (*solid bar* after exon 3). **b** Southern blotting analysis of genomic DNA from F1 offspring of chimera/wild-type backcrosses using the 3' probe just outside of the disrupted ACBP locus. **c** PCR analysis of genomic DNA from offspring of heterozygote/heterozygote intercrosses using 2 primer sets. (-/+) ACBP heterozygotes; (+/+) wild-type counterparts

Fig. 2.

Body weights of heterozygous ($-/+$) and wild-type ($+/+$) post-implantation embryo littermates and adult counterparts. Full body weights of wild-type (**a**, *solid bars*) and ACBP heterozygous (**a**, *cross-hatched bars*) post-implantation embryos are indicated at the E22 (14.5 dpc) and E25 (17.5 dpc) stages. Body weights of adult wild-type (**b**, *solid bars*) and ACBP heterozygous ACBP (**b**, *cross-hatched bars*) male and female counterparts. There were no significant differences in any of the groups

Fig. 3.

Genotypes of live F2 offspring from ACBP heterozygote male and female F1 intercrosses. From 21 intercross litters of mice, no homozygous ACBP-null offspring were detected. ACBP heterozygotes constituted 73.6% (126) of offspring (*cross-hatched bar*). Wild-type mice represented 26.4% [45] of offspring (*solid bar*). (-/-) homozygous ACBP-null; (-/+) ACBP heterozygotes; (+/+) wild-type mice from the same litters

Fig. 4.

Genotypes of post-implantation embryos from ACBP heterozygote intercrosses.

Embryos were examined at four stages of development: **a** E15 (9.5 dpc, 9 total embryos); **b** E19 (11.5 dpc, 8 total embryos); **c** E22 (14.5 dpc, 9 total embryos); and **d** E25 (17.5 dpc, 9 total embryos). (-/-) homozygous ACBP null (*open bars*); (-/+) ACBP heterozygotes (*cross-hatched bar*); (+/+) wild-type mice from the same litters (*black bar*). Values represent percent of total embryos for each genotype obtained at the indicated developmental stages

Fig. 5.

Pre-implantation development of homozygous ACBP-null and wild-type embryos. E1 (0.5-dpc) oocytes from ACBP heterozygote intercrosses were isolated, cultured in KSOM medium, and photographed every 12 h to monitor their development. At the indicated times, each embryo was genotyped by PCR. **a–d** show representative homozygous null ACBP pre-implantation embryos, while **e–h** show representative wild-type embryos at the same stages. Pre-implantation embryos at the E1 early oocyte stage (0.5 dpc, **a, e**) have *arrows* pointing to the two pronuclei, indicating fertilization; **b, f** indicate the E2 oocyte cleavage stage (1.5 dpc); **c, g**, the E3 morula stage (2.5 dpc); and **d, h** the E5 blastocyst stage (3.5 dpc). Degeneration of several embryos at the blastocyst stage probably represent dead/dying ACBP homozygous null embryos (**d**); DNA isolation was attempted numerous times but was too degenerated for definitive genotyping. (–/–) homozygous ACBP null; (–/+) ACBP heterozygotes; (+/+) wild-type mice

B

Genotypes	ACBP		m-calpain	
	2.5 dpc	3.0 dpc	2.5 dpc	3.0 dpc
(-/-)	-	∞	-	∞
(-/+)	+	+	-	+
(+/+)	+	+	-	+

Fig. 6.

Expression of ACBP and other key long-chain fatty acyl-CoA binding proteins in pre-implantation embryos. Embryos were isolated from ACBP heterozygote intercross mice, and levels of ACBP, sterol carrier protein-2 (SCP-2), sterol carrier protein-x (SCP-x), peroxisome proliferator-activated receptor- α (PPAR α), and m-calpain were determined by Q-rtPCR as described in "Methods". **a** *Acbp*, *Scp-2*, *Scp-x*, and *Ppara* expression was determined in homozygous ACBP-null embryos (*open bars*) and wild-type embryos (*solid bars*) at the 2.5-dpc morula stage (E3). Values represent means $\pm$ SEM; * $p < 0.05$ between ACBP-null and wild-type embryo; *** $p < 0.0001$ between ACBP-null and wild-type embryo. **b** *Acbp* and m-calpain expression was determined in homozygous null ACBP, heterozygous ACBP, and wild-type ACBP embryos at the morula (E3, 2.5 dpc) and early blastocyst (E4, 3.0 dpc) stages. (∞) indicates no live embryos found. (-/-) homozygous ACBP null; (-/+) ACBP heterozygous; (+/+) wild-type embryos

Table 1

Genotype distribution among pre-implantation embryos from heterozygote F1 intercrosses

Embryo Stage	Age (dpc)	Genotype					
		(-/-)		(-/+)		(+/+)	
		Total	%	Total	%	Total	%
Blastocyst (~32-cell)	3.5	0	0	81	63.3	47	36.7
Morula (~8-cell)	2.5	17	11.6	99	67.8	30	20.6
Oocyte (2-cell)	1.5	4	11.1	22	61.1	10	27.8

Genotyping was performed by PCR (*dpc* days post coitum)