ACYL CoA BINDING PROTEIN (ACBP) GENE ABLATION INDUCES PRE-IMPLANTATION EMBRYONIC LETHALITY IN MICE

A Thesis

by

DANILO LANDROCK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Biomedical Sciences

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Approved by:

Co-Chairs of Committee,	Ann B. Kier
	Friedhelm Schroeder
Committee Member,	Ian R.Tizard
Head of Department,	Linda L. Logan

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ABSTRACT

Acyl CoA Binding Protein (ACBP) Gene Ablation Induces Pre-Implantation Embryonic Lethality in Mice. (December 2010)

Danilo Landrock, Diplom (B.S. equivalent), Berlin, Germany

Co-Chairs of Advisory Committee, Dr. Ann B. Kier
Dr. Friedhelm Schroeder

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 result in any live homozygous deficient (null) $ACBP^{(-/-)}$ pups. Heterozygous and wild type embryos were detected at all postimplantation stages, but no homozygous ACBP null embryos were obtained– suggesting that an embryonic lethality occurred at a preimplantation 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) preimplantation 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 only at the earliest preimplantation 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.

ACKNOWLEDGMENTS

I would like to thank my committee chairs, Drs. Kier and Schroeder, and committee member, Dr. Tizard, for their continued support, guidance and encouragement throughout the course of this research.

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I would like to express my appreciation to the department faculty and staff for their tremendous support and making my experience at Texas A&M University a great one.

Thanks also to my parents for being there when most needed.

Finally, I would like to thank my wife and children. They have been my inspiration, my motivation and my drive.

NOMENCLATURE

Acaca	Acetyl CoA carboxylase A
Acacb	Acetyl CoA carboxylase B
Acadl	Long Chain Acyl CoA Dehydrogenase
ACBP	Acyl CoA Binding Protein
Atp1b1	Na^+, K^+-ATPase (ATPase, Na^+/K^+ transporting beta 1 polypeptide)
Atp2a2	Ca^{++}-ATPase (endoplasmic reticulum)
Capn2	m-Calpain
CoA	Coenzyme A
CPT1	Carnitine Palmitoyltransferase 1
Dbi	Diazepam Binding Inhibitor
DNA	Deoxyribonucleic acid
dpc	Days post coitum
EDTA	Ethylenediaminetetraacetic acid
ES cells	Embryonic Stemm Cells
FABP1	Fatty Acid Binding Protein 1 (liver fatty acid binding protein, L-FABP)
FABP2	Fatty Acid Binding Protein 2 (intestinal fatty acid binding protein, I-FABP)
FABP3	Fatty Acid Binding Protein 3 (heart/muscle fatty acid binding protein, H-FABP)
FABP4	Fatty Acid Binding Protein 4 (adipocyte fatty acid binding protein, A-FABP)

FABP5	Fatty Acid Binding Protein 5 (epidermal/keratinocyte fatty acid binding protein, E-FABP)
FABP6	Fatty Acid Binding Protein 6 (ileal fatty acid binding protein, Il-FABP)
FABP7	Fatty Acid Binding Protein 7 (brain fatty acid binding protein, B-FABP)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gck	Glucokinase (hexokinase 4)
HCG	Human Chorionic Gonadotropin
Hk1	Hexokinase 1
Hk2	Hexokinase 2
Hk3	Hexokinase 3
Hmgcr	HMG-CoA reductase
kDa	Kilo Dalton
KSOM	Potassium Simplex Optimized Medium
LCFA	Long Chain Fatty Acids
LIF	Leukemia Inhibitory Factor
Lipe	Hormone Sensitive Lipase
PCR	Polymerase Chain Reaction
PMEF	Primary Mouse Embryonic Fibroblasts
PMSG	Pregnant Mare Serum Gonadotropin
Prkaa2	Protein Kinase AMP activated α 2 catalytic subunit
PPARα	Peroxisome Proliferator Activated Receptor α
PPARβ	Peroxisome Proliferator Activated Recepto β
PPARγ	Peroxisome Proliferator Activated Receptor γ

Q-rtPCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic acid
SCP-x	Sterol Carrier Protein x
SCP-2	Sterol Carrier Protein 2
SDS	Sodium Dodecyl Sulfate
Slc25a4	Solute Carrier Family 25 member 4 (mitochondrial carrier, adenine nucleotide translocator)
Slc25a5	Solute Carrier Family 25 member 5 (mitochondrial carrier, adenine nucleotide translocator)
Slc25a13	Solute Carrier Family 25 member 13 (mitochondrial carrier, adenine nucleotide translocator)
Slc25a20	Solute Carrier Family 25 member 20 (mitochondrial carnitine/acylcarnitine translocase)
Slc25a31	Solute Carrier Family 25 member 31 (mitochondrial carrier, adenine nucleotide translocator)
SREBP	Sterol Regulatory Element Binding Protein
Thrα	Thyroid hormone receptor α
Thrβ	Thyroid hormone receptor β

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INTRODUCTION AND LITERATURE REVIEW*

Known Physiological Role of Acyl CoA Binding Protein

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 (<10nM K_ds) 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, 10-13). For example, LCFA-CoAs at very low concentration (K_i <50nM) inhibit acetyl CoA carboxylase (Acac), the key rate limiting enzyme of de novo fatty acid synthesis from glucose. By binding LCFA-CoA, ACBP removes this end-product inhibition to stimulate Acac (10). Likewise, increased serum levels of LCFAs resulting in high intracellular LCFA-CoAs are deleterious to multiple cell functions and are associated with diabetes and obesity (10, 14). Again, by binding and reducing the unbound levels of LCFA-CoAs, ACBP plays important roles not only in normal regulation of LCFA-CoA

This thesis follows the style of Lipids.

transport, metabolism, signaling, vesicular trafficking, and nuclear regulation, but also in opposing the deleterious effects of elevated intracellular LCFA-CoA levels (10, 14). The physiological relevance of these findings is supported mainly by studies of ACBP overexpression in yeast, mice, rats, and plants (*Arabidopsis*) (15-17). In vivo, pancreatic insulin secretion is affected by LCFA-CoA and ACBP levels as well as by glucose (14, 18, 19). ACBP overexpressing rats fed a medium chain fatty acid-rich diet exhibited improved glucose tolerance and lower serum insulin levels (17). A single nucleotide polymorphism in the promoter region of the human ACBP gene is associated with reduced risk of type 2 diabetes in two German study populations—likely due to increased transcriptional activity of ACBP (20).

Hypothesis

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. While deletion of the ACBP gene in yeast results in a slower growing phenotype, the yeast subsequently adapt to a faster growing phenotype (21). A conditional ACBP inhibition knock-down in yeast alters lipids, membranes, and vesicle accumulation, but is not lethal (21). Although disruption of the 10 kDa ACBP gene in the plant *Arabidopsis* is not lethal, *Arabidopsis* expresses at least 5 additional ACBP genes that also bind LCFA-CoA—likely compensating for the loss of the 10 kDa ACBP6 (22, 23). In contrast to plants, mice and humans have only a single ACBP encoding gene (7, 24). Treatment of mouse 3T3-L1 adipocytes with ACBP antisense RNA inhibits differentiation (25), while treatment of several human cell lines (HeLa, HepG2, Chang) with small interference

RNAi results in lethality (26). However, because transformed cells are often deficient in the other LCFA-CoA binding proteins (1, 27-29) it is difficult on this basis alone to predict if ACBP is an essential protein in mammals.

Purpose of Study

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

MATERIALS AND METHODS*

Construct Preparation

A BAC clone containing the ACBP sequence was obtained from the BACPAC Resource Center (Oakland, CA). 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), Potasium 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 hour light/dark cycle in a temperature-controlled facility (25°C), access to standard commercial rodent chow (Teklad®, Harlan, Indianapolis, IN) 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 mouse ACBP null targeting construct was designed to replace the N-terminal promoter region (including the known SREBP and PPARγ 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) were used to form the backbone of the ACBP gene targeting construct: a 7 kbp *Xho*I 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. Mouse ACBP genomic sequences were confirmed by extensive restriction mapping and DNA sequencing. The 5' arm of homology was generated by ligating a 4.2 kbp *Xho*I/*Sma*I fragment from the 7 kbp *Xho*I clone into pBlueScript-SK (pBS-SK; Stratagene, La Jolla,

CA). An intermediate targeting construct consisting of the neomycin resistance marker and a 3.9 kbp *Xho*I/*Hin*dIII fragment from the 7 kbp *Hin*dIII clone was generated by ligating the neo cassette from a pPGK-Neo vector to the 3.9 kbp *Xho*I/*Hin*dIII 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 *Hin*dIII, 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. 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 offspring 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.

Isolation of Pre- and Postimplantation Stage Embryos

ACBP heterozygotes or wild-type (control) females were paired overnight with ACBP heterozygotes and 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. Postimplantation 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. Preimplantation 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 preimplantation embryos at 37°C, 5% CO_2. One-cell stage embryos were treated with 1mg/ml of hyaluronidase and subsequently rinsed 5-10 times in sterile M2 medium to remove cumulus cells. The development and morphology of preimplantation stages were monitored by visualizing the embryos with an inverted phase contrast microscope (Nikon Diaphoto 300, Nikon, Tokyo, Japan) at 12-hour intervals after oocyte collection.

Genotyping of Pre- and Postimplantation Embryos

Genotyping of individual preimplantation 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 postimplantation embryos was obtained by digesting a small section of tail in 500µl of lysis buffer (10mM Tris-HCl, 1mM EDTA, 300mM Na acetate, 1% SDS, 0.2mg/ml proteinase K) for 6 hours at 55ºC, followed by 20 minutes at 95ºC 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) according to manufacturer's protocol. Expression patterns were analyzed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) 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 minutes for reverse transcription prior to amplification, 95°C for 10 minutes before the first cycle, 95°C for 15 seconds, and 60°C for 1 minute, repeated 60 times. For other specific probes and primers, TaqMan® Gene Expression Assay products for mouse peroxisome proliferator-activated receptor-α

(Pparα, 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 Pparα, Scp-2, Scp-x, and Capn2 in homozygous null preimplantation embryos were normalized to a housekeeping gene (18S rRNA) and were made relative to the control wild-type preimplantation embryos and were calculated using the comparative $2^{-\Delta\Delta C_T}$ method (30) where $\Delta\Delta C_T = [C_T \text{ of target gene} - C_T \text{ of 18s}]_{\text{ACBP-null embryos}} - [C_T \text{ of target gene} - C_T \text{ of 18s}]_{\text{wild type embryos}}$ as described in User Bulletin 2, ABI PRISM 7000 SDS.

Calculation of Free, Unbound Long Chain Fatty Acyl CoA

Because high levels of free LCFA-CoA may inappropriately affect proteins or act as potent detergents (31, 32), the effect of ACBP level on free unbound LCFA-CoA concentration was modeled as a function of ACBP versus total LCFA-CoAs. Calculation of free and unbound LCFA-CoA as a function of total LCFA-CoA and ACBP concentration was performed using Eq. 1,

$$[C_T] = [P]\left(\frac{[C_F]}{K_d + [C_F]}\right) + [C_F] \qquad \text{Eq. 1}$$

where C_T and C_F is the total and free acyl CoA concentration respectively, P is the ACBP

concentration, and K_d is the dissociation constant of ACBP. C_F was plotted vs. C_T. Since the affinity of ACBP's single binding site for LCFA-CoAs as previously determined by fluorescence, microcalorimetry, and mass spectrometery was in a narrow range (K_d = 7-12 nM) (33-35), an average K_d = 9.5 nM was used in this calculation. The range of LCFA-CoA concentration used in these calculations was based on the known range reported for all tissues, i.e. 5-150 μM (31, 32).

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 (19, 36, 37). 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). Therefore, the mouse ACBP null targeting construct was designed to replace the N-terminal promoter region (including the known SREBP and PPARα 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 immediately after the disrupted locus (horizontal solid bar after exon 3). 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.

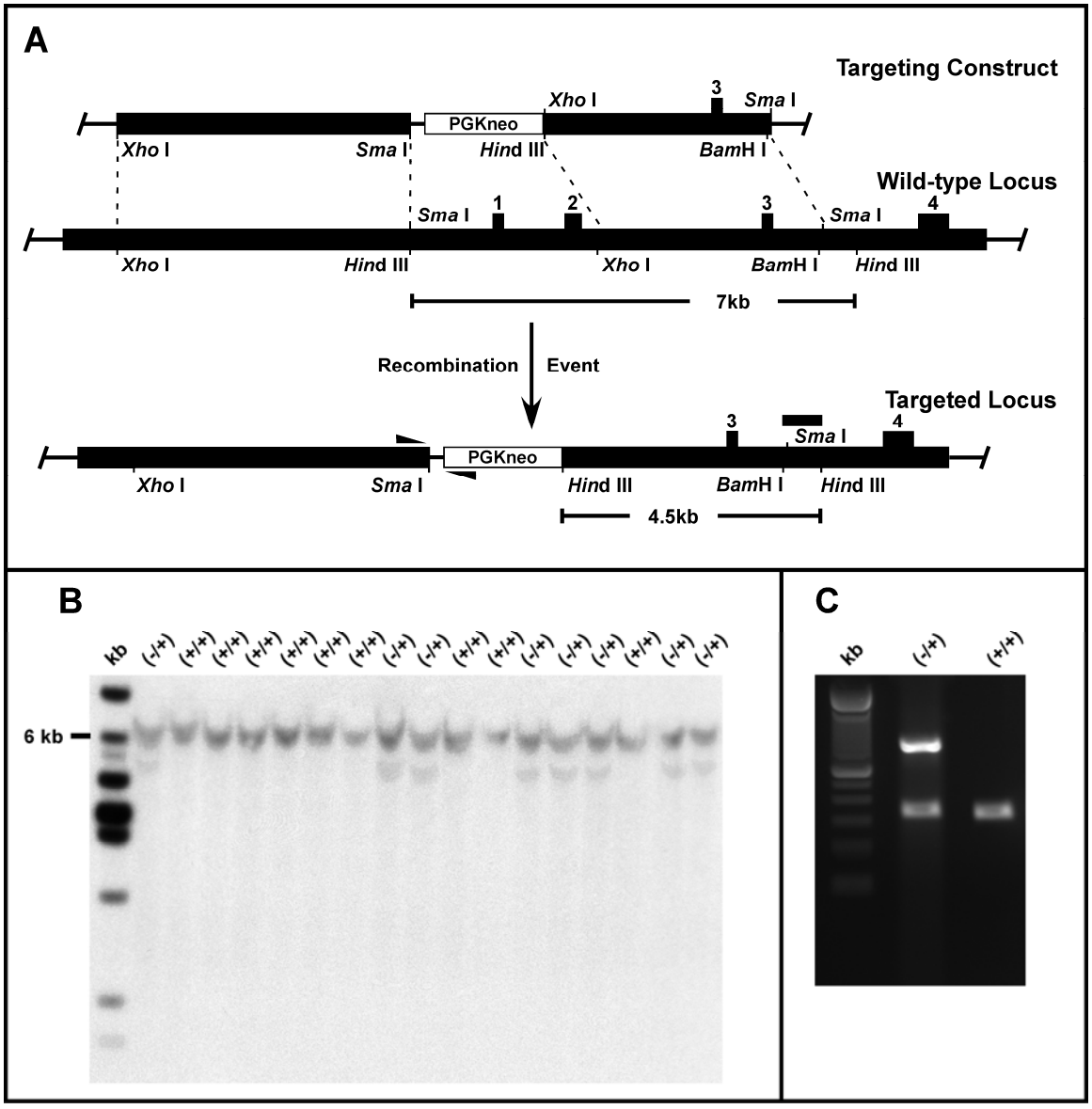


Fig. 1. Construct Design and Screening of ACBP Gene-Ablated Mice. (A) The targeting construct, wild-type ACBP locus and targeted locus after homologous recombination. Two lower diagrams show the wild-type locus and targeted ACBP locus with expected *Hin*dIII fragment sizes with the indicated probe (solid 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.

ACBP Heterozygous Mice are Phenotypically Normal

Wild type and heterozygous mice were indistinguishable with respect to visual appearance, postimplantation embryonic weight (Fig. 2A), body weight at weaning (not shown), adult body weight (Fig. 2B), and fertility (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 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.

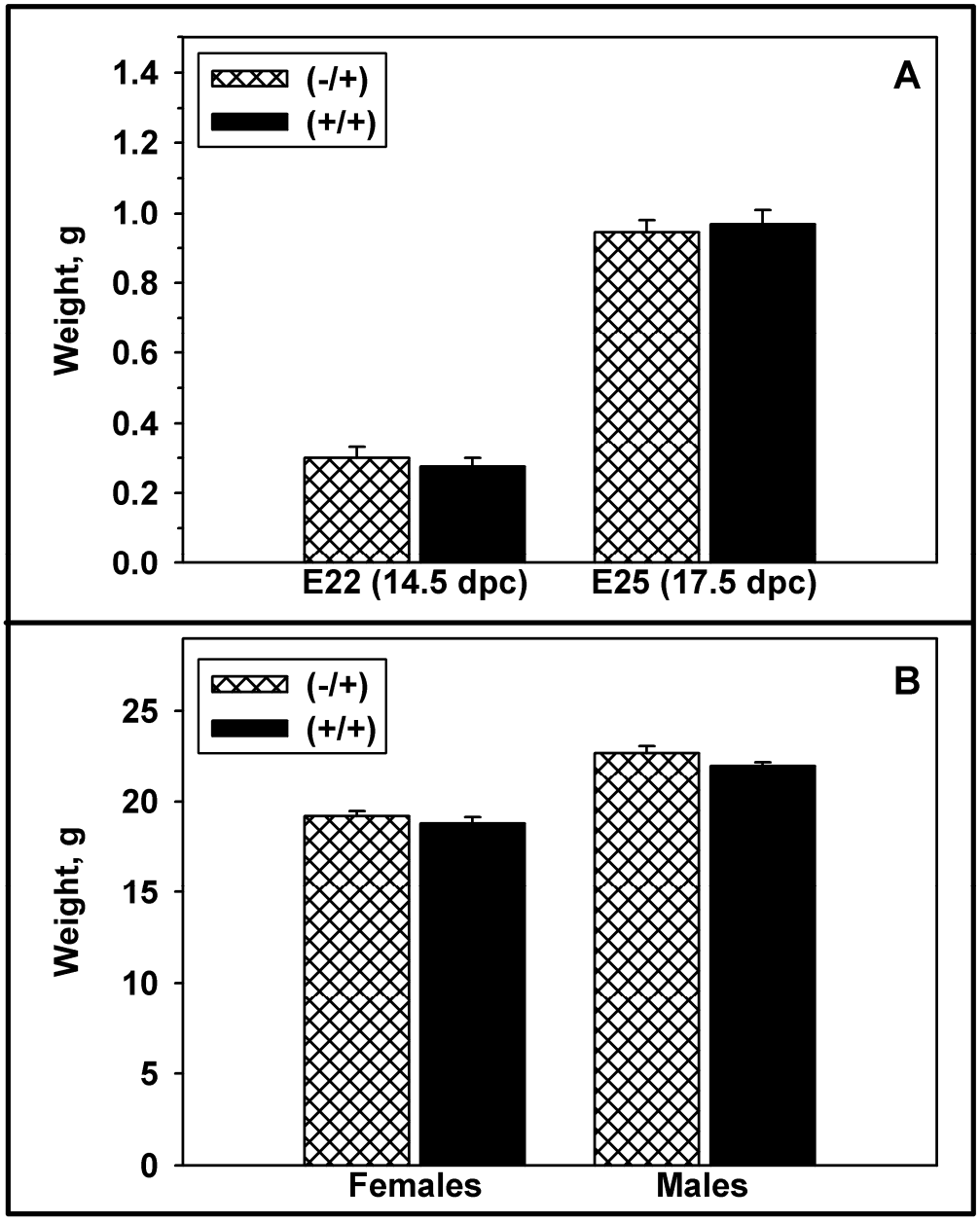


Fig. 2. Body Weights of Heterozygous (-/+) and Wild-Type (+/+) Postimplantation Embryo Littermates and Adult Counterparts. Full body weights of wild-type (A, solid bars) and ACBP heterozygous (A, cross-hatched bars) postimplantation embryos are indicated at the E22 (14.5 dpc) and E25 (17.5 dpc) stages. 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.

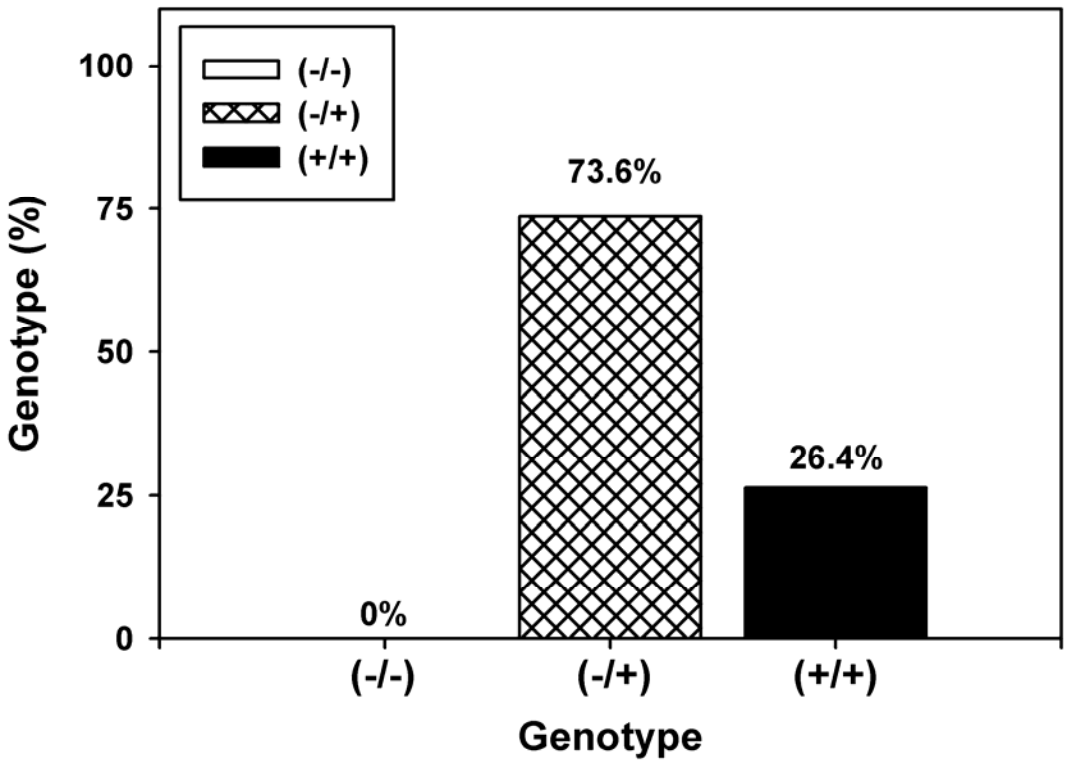


Fig. 3. Genotypes of Live F2 Offspring from ACBP Heterozygote Male and Female F1 Intercrosses. From 21 intercross litters of mice, no homozygous ACBP null offspring were detected. ACBP heterozygotes constituted 73.6% (126) of offspring (cross-hatched bar). Wild-type mice represented 26.4% (45) of offspring (solid bar). (-/-) = homozygous ACBP null; (-/+) = ACBP heterozygotes; (+/+) = wild-type mice from the same litters.

Genotypes of Postimplantation Embryos

To determine if ACBP gene ablation resulted from a postimplantation embryonic lethality, the genotypes of postimplantation F2 embryos from F1 ACBP heterozygote/heterozygote intercrosses were determined. Of the 35 postimplantation 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 Preimplantation Embryos

To determine if ACBP null mice resulted in a preimplantation embryonic lethality, the genotypes of 310 of 510 preimplantation 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 likely represented ACBP homozygous null embryos. Thus, ACBP gene ablation resulted in early preimplantation 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.

Table 1. Genotype Distribution Among Preimplantation Embryos from Heterozygote F1 Intercrosses.

Embryo		Genotype					
Stage	Age (dpc)	(-/-)		(-/+)		(+/+)	
		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).

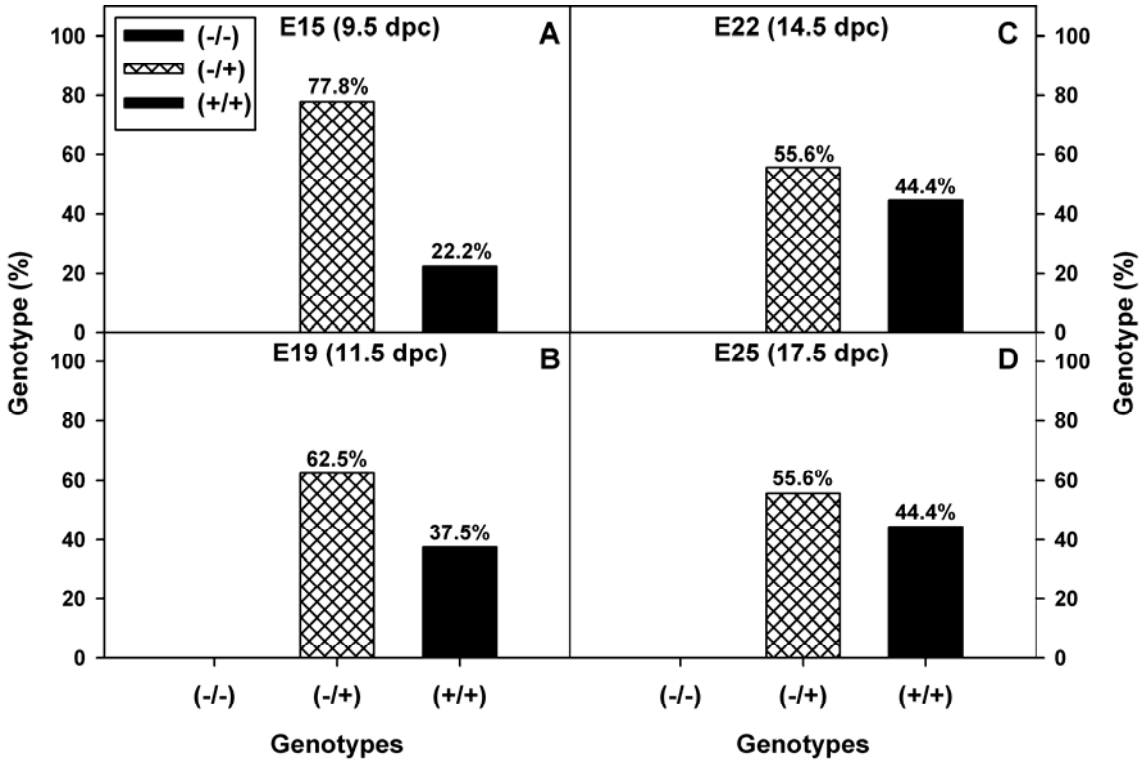
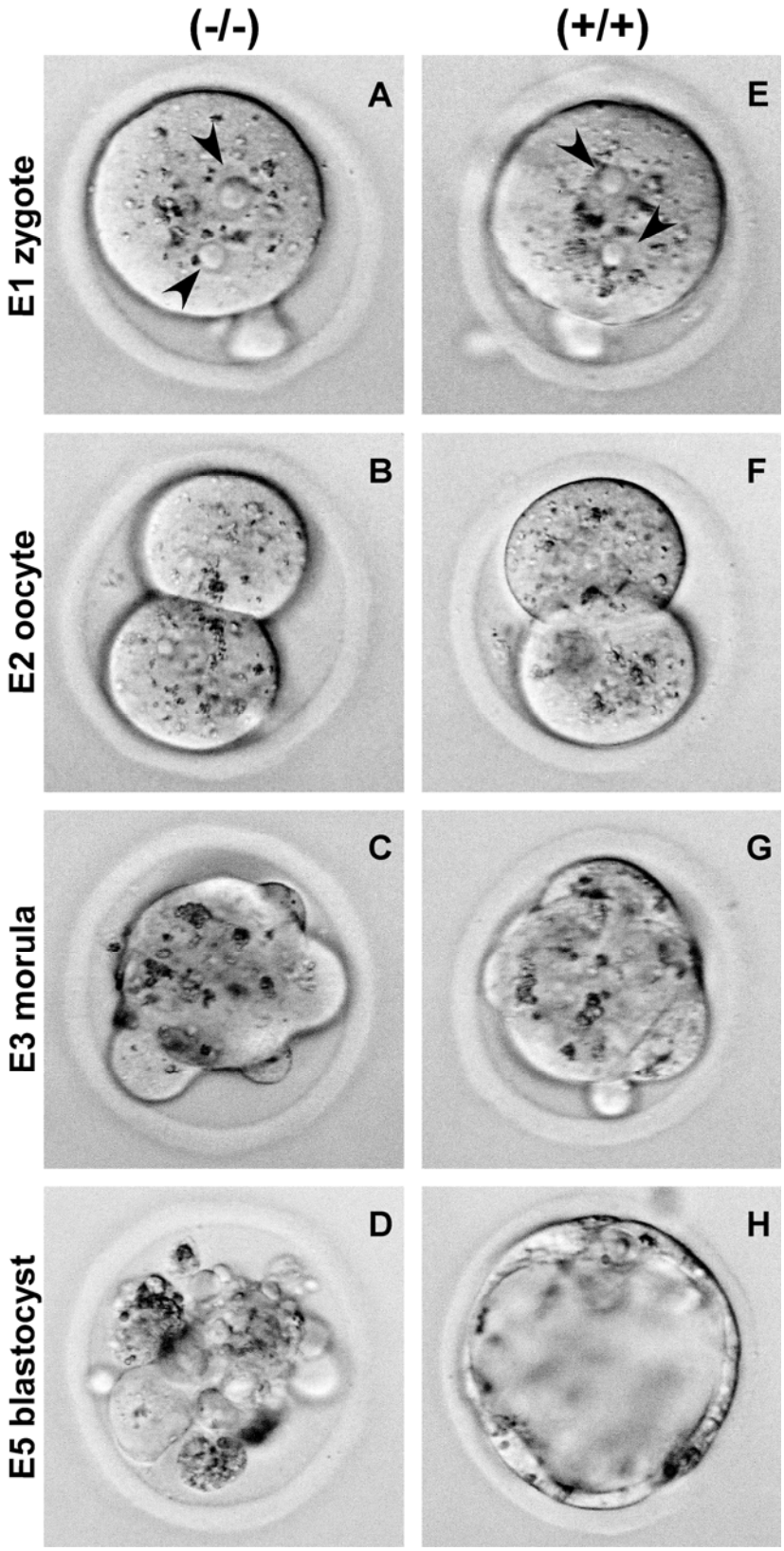


Fig. 4. Genotypes of Postimplantation Embryos from ACBP Heterozygote Intercrosses. Embryos were examined at four stages of development: (A) E15 (9.5 dpc, 9 total embryos); (B) E19 (11.5 dpc, 8 total embryos); (C) E22 (14.5 dpc, 9 total embryos); and (D) E25 (17.5 dpc, 9 total embryos). (-/-) = homozygous ACBP null (open bars); (-/+) = ACBP heterozygotes (cross-hatched bar); (+/+) = wild-type mice from the same litters (black bar).

Fig. 5. Preimplantation 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 12h to monitor their development. At the indicated times, each embryo was genotyped by PCR. Panels A-D show representative homozygous null ACBP preimplantation embryos, while panels E-H show representative wild-type embryos at the same stages. Preimplantation embryos at the E1 early oocyte stage (0.5 dpc, panels A, E)) have arrows pointing to the two pronuclei, indicating fertilization; Panels B, F indicate the E2 oocyte cleavage stage (1.5 dpc); panels C, G, the E3 morula stage (2.5 dpc); and panels D, H the E5 blastocyst stage (3.5 dpc). Degeneration of several embryos at the blastocyst stage likely 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.


(-/-)
(+/+)
E1 zygote
E2 oocyte
E3 morula
E5 blastocyst
A
B
C
D
E
F
G
H

Q-rtPCR of ACBP in Preimplantation Embryos

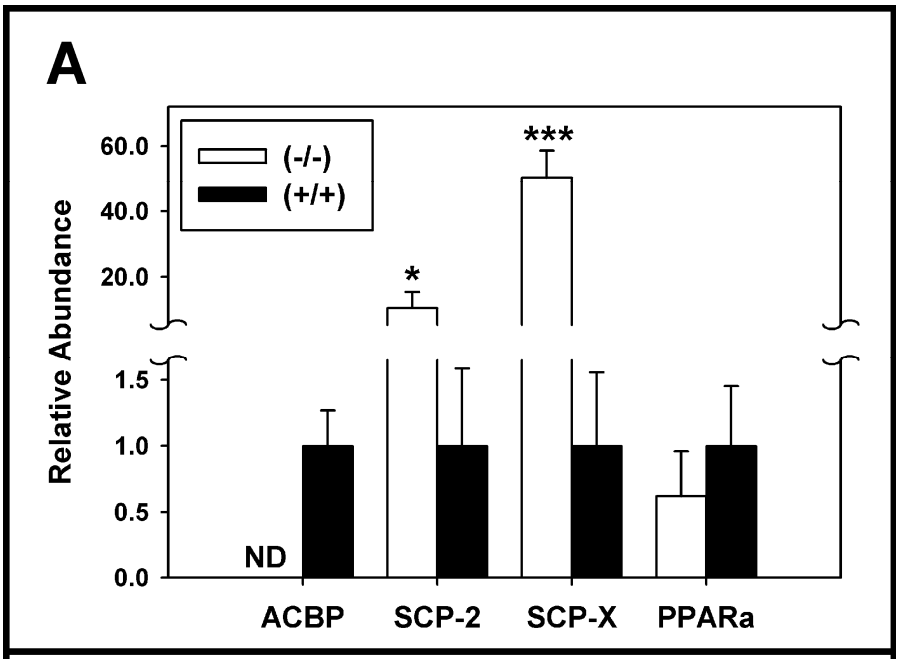
To determine if the lethality was associated with total absence of ACBP transcription, 2.5 and 3.0 dpc embryos from ACBP heterozygote intercrosses were examined by Q-rtPCR. The ACBP transcript was detected in wild-type and heterozygous embryos at the 2.5 dpc morula (Fig. 6A, 6B) and 3.0 dpc stage (Fig. 6B) just prior to blastocyst formation. In contrast, no Acbp was detected in the morula 2.5 dpc stage of ACBP null embryos (Fig. 6A, 6B). Detection of Acbp 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 (Fig. 5D, Fig. 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, 38); their ablation is not lethal (39-41); and they are expressed as early as the zygote 1-cell stage, slightly earlier than ACBP at the 2-cell stage (Fig. 7A). SCP-2 expression in null ACBP 2.5 dpc morulae was upregulated 10-fold (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 (PPARα) was also investigated. PPARα exhibits high affinity for LCFA-CoAs (12, 13) and PPARα coactivator recruitment and transcriptional activity are regulated by both LCFA-CoAs and ACBP (12, 13, 42, 43). Furthermore, expression of the PPARα transcript occurred by the zygote (1-cell) stage, thereby preceding that appearance of ACBP which normally occurs at the oocyte cleavage (2-cell) stage (Fig. 7A). However, ACBP gene ablation did not significantly alter the expression of Pparα at the morula (2.5 dpc) stage. Thus, viability of very early preimplantation ACBP null embryos was not associated with concomitant upregulation of PPARα.



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 Preimplantation 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 (PPARα), and m-calpain were determined by Q-rtPCR as described in Methods. (A) Acbp, Scp-2, Scp-x, and Pparα 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 ± 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.

Since ACBP is a potent activator of m-calpain, a protease involved in apoptosis (44), the possibility that m-calpain transcript (Capn2) is expressed at an early preimplantation 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 preimplantation 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_is as low as 50 nM) of many enzymes, transporters, and receptors involved in lipid and glucose metabolism (Table 2). 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. At the upper range of physiological ACBP concentration (50 μM), ACBP very effectively buffered the unbound LCFA-CoA concentrations over a relatively broad range (Fig. 8A). The unbound LCFA-CoA concentration was <50 nM up to 40 μM total LCFA-CoA (Fig. 8B). In contrast, estimated lower physiological ACBP concentration (6 μM) was relatively ineffective in buffering the unbound LCFA-CoA level (Fig. 8A), which was > 50 nM even at 10 μM total LCFA-CoA (Fig. 8B). Taken together, these data suggest that the loss of ACBP would likely 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.

Fig. 7. Embryonic Development Timeline of the First Detected Expression of Key Fatty Acid Binding Proteins (A), Nuclear Receptors (A), and Enzymes (B) Involved in Lipid and Glucose Metabolism and Regulated by Low Levels of LCFA-CoAs. Transcript abbreviations encoding the respective proteins: Acbp, ACBP; Scp-x, SCP-x; Scp-2, SCP-2; Fabp1, L-FABP; Fabp2, I-FABP; Fabp3, H-FABP; Fabp4, A-FABP; Fabp5, E-FABP; Fabp7, B-FABP; Ppara, PPARα; Pparg, PPARγ; Thra, thyroid hormone receptor-α; Thrb, thyroid hormone receptor-β; Capn2, m-calpain; Acaca, acetyl CoA carboxylase A; Acacb, acetyl CoA carboxylase B; Prkaa2, AMP-activated kinase (protein kinase, AMP-activated, alpha 2 catalytic subunit); Hmgcr, HMG-CoA reductase; Slc25a4, solute carrier family 25 member 4 (mitochondrial carrier, adenine nucleotide translocator); Slc25a5, solute carrier family 25 member 4 (mitochondrial carrier, adenine nucleotide translocator); Slc25a13, solute carrier family 25 member 13 (mitochondrial carrier, adenine nucleotide translocator); Slc25a20, solute carrier family 25 member 20 (mitochondrial carrier, adenine nucleotide translocator); Slc25a31, solute carrier family 25 member 31 (mitochondrial carrier, adenine nucleotide translocator); Atp1b1, Na^{+},K^{+}-ATPase (ATPase, Na+/K+ transporting beta 1 polypeptide); Atp2a2, Ca^{++}-ATPase (endoplasmic reticulum); Hk1, hexokinase 1; Hk2, hexokinase ; Hk3, hexokinase 3; Gck, glucokinase (hexokinase 4); Acadl, long chain acyl CoA dehydrogenase; and Lipe, hormone sensitive lipase. Expression profiles were established by Q-rtPCR(*), or inferred from Expressed Sequence Tag (EST) counts, from the NCBI UniGene database, October 2009, http://www.ncbi.nlm.nih.gov/unigene).

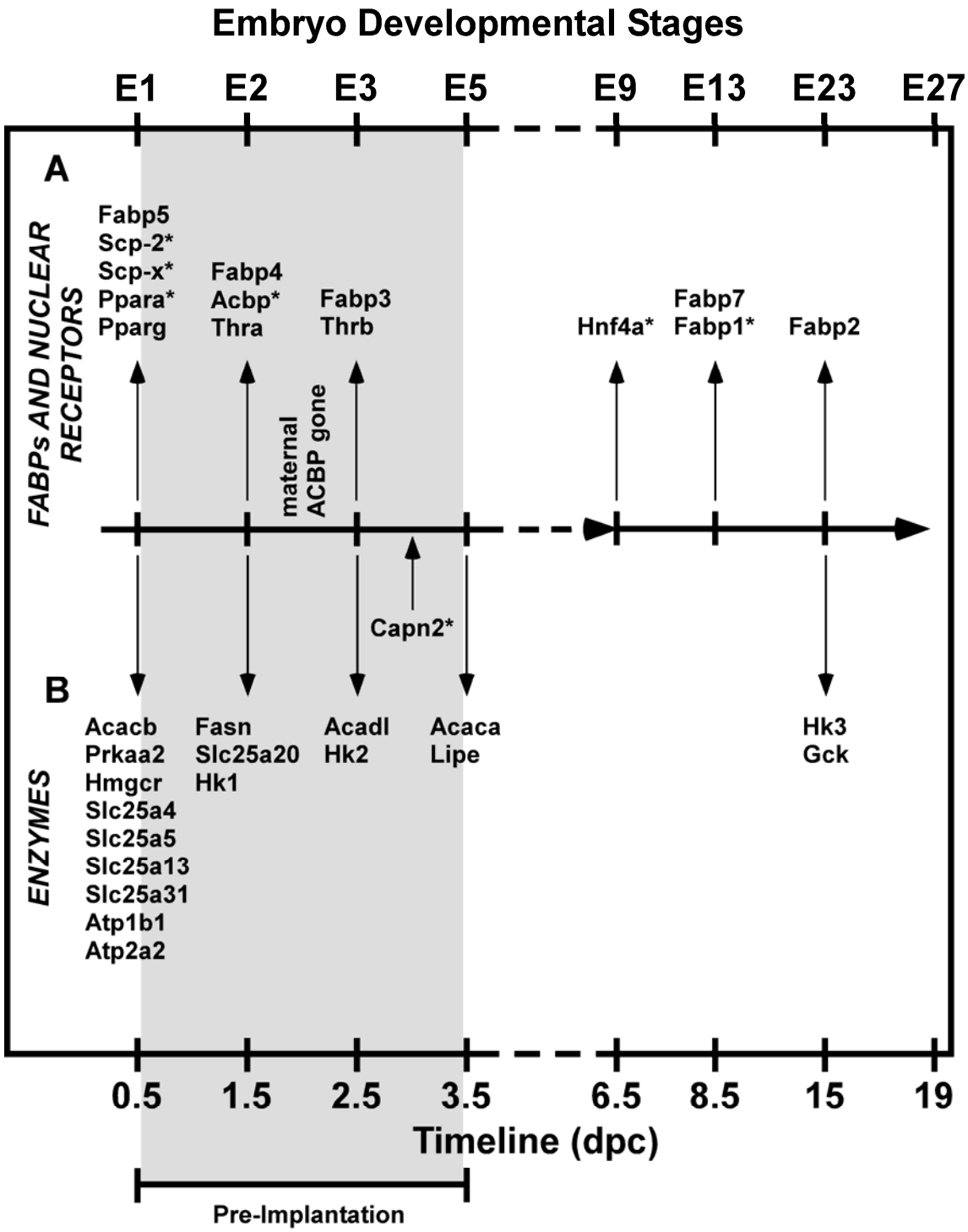

Embryo Developmental Stages
E1
E2
E3
E5
E9
E13
E23
E27
A
FABPs AND NUCLEAR RECEPTORS
Fabp5
Scp-2*
Scp-x*
Ppara*
Pparg
Fabp4
Acbp*
Thra
Fabp3
Thrb
maternal ACBP gone
Hnf4a*
Fabp7
Fabp1*
Fabp2
Capn2*
B
ENZYMES
Acacb
Prkaa2
Hmgcr
Slc25a4
Slc25a5
Slc25a13
Slc25a31
Atp1b1
Atp2a2
Fasn
Slc25a20
Hk1
Acadl
Hk2
Acaca
Lipe
Hk3
Gck
0.5
1.5
2.5
3.5
6.5
8.5
15
19
Timeline (dpc)
Pre-Implantation

Table 2. Regulation of Key Enzymes/Receptors in Lipid and Glucose Metabolism by LCFA-CoAs and ACBP.

Gene	Protein	Effect of LCFA-CoA	K_i (μM)	Effect of ACBP	References
Fatty Acid Synthesis					
Acaca	Acetyl CoA carboxylase	Inhibit	0.05	Stimulate	(31)
	Mitchondrial acyl CoA synthetase	Inhibit	4	Stimulate	(45)
Fatty Acid and Cholesterol Synthesis					
Prkaa2	AMP-activated kinase kinase	Stimulate	nM	?	(46)
Cholesterol Synthesis					
Hmgcr	HMG-CoA reductase	Inhibit	<2	?	(47)
Fatty Acid β-Oxidation					
Cpt1	Carnitine palmitoyl transferase I	Inhibit	20	Stimulate	(48)
Acadl	Long chain acyl CoA dehydrogenase	Inhibit	0.2	?	(49)
Cholesteryl ester/Triacyglyceride Hydrolysis					
Lipe	Hormone sensitive lipase	Inhibit	0.5	?	(50)
Glucose Metabolism					
Gck (Hk4)	Glucokinase	Inhibit	0.5	?	(51)
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Inhibit	0.5	?	(52)
Slc25a	Adenine nucleotide translocase	Inhibit	<1	Stimulate	(31)
Receptors, Transporters, and Processes					
Thr	Nuclear thyroid hormone receptor	Inhibit	0.1	?	(53)
Atp1b1	Na^+,K^+-ATPase (plasma membrane)	Stimulate	3	?	(54)
Atp2a2	Ca^{++}-ATPase (endoplasmic reticulum)	Stimulate	<0.5	?	(55)
	Vesicular transport (Golgi)	Stimulate		?	(56)

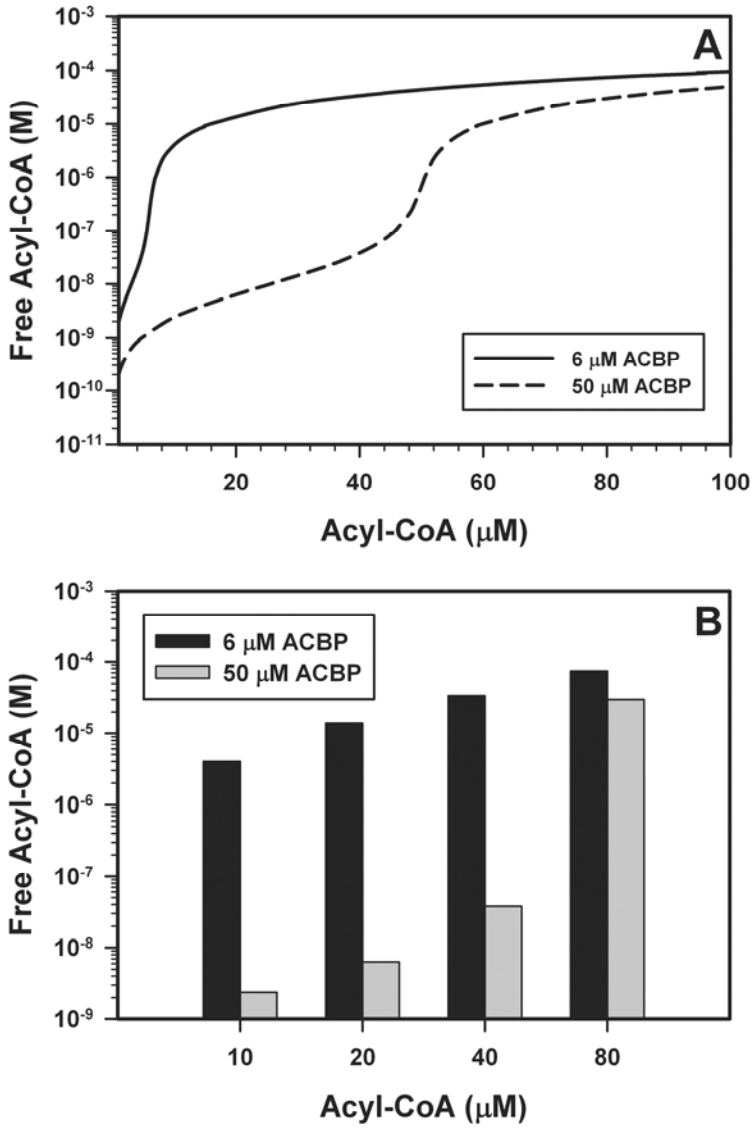


Fig. 8. Modeling the Effect of ACBP Ligand Binding on Free LCFA-CoA Concentration in Embryos. Free LCFA-CoA concentrations were calculated at a constant ACBP level in the presence of increasing total LCFA-CoA level as described in the Methods, using the average dissociation constant of ACBP, K_d = 9.6 nM. Panel A, amount of free unbound LCFA-CoA as a function of total available LCFA-CoA in the range of 1-100 μM was plotted for embryos containing physiologically high (50 μM) and low (6 μM) ACBP. Panel B, Vertical bar graph of the effect of physiologically high (50 μM) and low (6 μM) ACBP concentration on amount of total LCFA-CoAs of 10, 20, 40, and 80 μM.

SUMMARY AND 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, 14, 57). While physiologic tissue total LCFA-CoA levels are in the 5-150 μM range (1, 10, 14, 57), 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 (Table 2). The respective K_is 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, 14, 57). 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 ACBP (reviewed in 1, 10, 14, 57). The present investigation determining the effect of ACBP gene ablation on the phenotype of mice yielded the following insights:

ACBP - First Embryonic Lethal Mutation of the LCFA-CoA Binding Proteins

First, single gene deletion of ACBP is the first known embryonic lethal mutation of 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

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LCFA-CoAs with high affinity in the order: acyl CoA binding protein (ACBP) (1, 10, 14, 57) > sterol carrier proteins [sterol carrier protein-2 (SCP-2), sterol carrier protein-x (SCP-x)] (58) > select fatty acid binding proteins [FABP1 (L-FABP) >> FABP7, FABP2, FABP3 (B-FABP, I-FABP, H-FABP)] (59-61). Of these LCFA-CoA binding proteins only ACBP, SCP-2/SCP-x, and FABP3 are expressed in preimplantation embryos (Figure 7.). However, FABP3 (H-FABP) binds LCFA-CoA more weakly than FABP1 (L-FABP)—a protein not expressed in preimplantation embryos (Figure 7.) (59, 60). Thus, redundancy in LCFA-CoA binding proteins is much more limited in pre-implantation embryos than in mature tissues. This would suggest that loss of one of the lower affinity LCFA-CoA binding proteins would not likely 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 Fapb7 (brain B-FABP) (62-64). Likewise, ablation of SCP-2 and SCP-x is not lethal (40, 41, 65). Even intercrossing of L-FABP null mice and SCP-2/SCP-x null mice does not result in lethality (66). Thus, embryonic lethality in mice ablated for the single gene encoding ACBP is unique among the multiple LCFA-CoA binding proteins known to exist in cytoplasm of mammalian tissues.

ACBP Gene Ablation–Induced Lethality at Very Early Preimplantation Stages

Second, the ACBP gene ablation-induced embryonic lethality at very early preimplantation stages where the embryo is not well differentiated was consistent with that observed in highly undifferentiated tumor cells wherein ACBP is reduced by

treatment with ACBP antisense RNA. Transformed cell lines express ACBP, but have markedly reduced levels of other LCFA-CoA binding proteins (e.g. FABPs, SCP-2/SCPx) (reviewed in 1, 27-29). Consequently, ACBP antisense RNA treatment of highly undifferentiated human transformed cell lines is lethal–likely due to loss of LCFA-CoA buffering capacity concomitant with loss of ACBP (3, 26). The fact that some apparently normal ACBP null embryos, albeit reduced in expected numbers, were still alive at the 1-8 cell preimplantation stages, but not later embryonic stages, was most likely due to the presence of residual maternal ACBP protein. Although almost all maternal mRNA is degraded by the middle of the 2-cell stage (67, 68), maternal ACBP protein likely survives longer as indicated by the relatively long the $t_{1/2}$ of ACBP protein in the range of 25-53 hours (67-69). Concomitant upregulation of two much 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.

Lethality of ACBP Ablation Consistent With Deleterious Effects of LCFA-CoAs

Third, the early preimplantation 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 triglyceride) are the major energy source in unfertilized oocytes, thereafter very little energy is derived from fatty acids and intracellular triglyceride is maintained relatively constant (70). Since there is too little glycogen to sustain preimplantation embryos, especially that of the mouse, the

preimplantation embryo is heavily dependent on exogenous energy supply (70). Pyruvate and lactic acid in oviductal fluid represent the major carbon sources of mouse preimplantation embryos up to the 4 cell stage, while glucose serves this purpose thereafter (70-72). Acetyl CoA derived from these nutrients is oxidized to produce energy or is used as a substrate for the cytoplasmic enzyme acetyl CoA carboxylase (Acac) which catalyzes the rate limiting step in fatty acid biosynthesis for formation of membranes and longer term energy storage than can be accomplished by glycogen. Acetyl CoA carboxylase is exquisitely sensitive (K_i 50nM) to the presence of even very low levels of LCFA-CoA (10). 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 because the other major LCFA-CoA binding protein (FABP1, i.e. L-FABP) is not induced until much later in development. Furthermore, LCFA-CoA binding proteins such as SCP-2 and SCP-x (present and concomitantly upregulated in ACBP null preimplantation embryos) are largely compartmentalized in peroxisomes and are present at much lower level (reviewed in 58). Since ACBP is thought to be the most effective of the LCFA-CoA binding proteins in buffering the unbound free LCFA-CoA concentration (10, 73), complete loss of ACBP would thus be expected to greatly increase unbound free LCFA-CoAs over that normally present in cells and tissues.

ACBP Exhibits a Unique Role in Early Preimplantation Embryonic Development

Fourth, ACBP exhibits a unique role in early preimplantation 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 (Fig. 7), but gene ablation of many of these proteins is not been lethal (40, 41, 62-65). ACBP is also known to enter the nucleus, bind nuclear receptors (PPARα, PPARγ), and regulate transcriptional activity of these receptors (11, 29, 74, 75). PPARα itself exhibits high affinity for and is regulated by LCFA-CoAs transported by ACBP (12, 13, 29). While PPARs α and γ are both present at the zygote one-cell stage (Fig. 7), ablation of PPARα is not lethal, and ablation of PPARγ is lethal but not until much later, i.e. the post-implantation E10 stage (76, 77). Taken together, these findings suggest that complete absence of ACBP may also dysregulate PPARs–nuclear transcription factors important in development as well as lipid and glucose metabolism (78).

ACBP Is an Essential Protein in Mammals

Finally, ACBP does not appear to be an essential protein in other eukaryotes since ACBP deletion results in the appearance of revertants (yeast) and likely compensation by 5 other ACBP genes in plants (*Arabidopsis*) (21-23, 79). In contrast, mice and humans have only a single ACBP encoding gene (7, 24), which may explain why ACBP single gene ablation in mice was lethal.

Conclusion

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. Although several other cytosolic LCFA-CoA binding proteins are expressed at these early pre-implantation embryonic stages, ablation of these other cytosolic LCFA-

CoA binding proteins 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 preimplantation embryonic development remains to be identified, at least two general possibilities may be considered: (i) 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 likely 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) required for normal glucose metabolism and fatty acid synthesis in these rapidly growing preimplantation embryos; (ii) Since ACBP normally interacts with and regulates several nuclear receptors (e.g. PPARs) present in early preimplantation 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 preimplantation ACBP null embryos.

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