A Knock-out Mouse Model for Methylmalonic Aciduria Resulting in Neonatal Lethality*

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Methylmalonic aciduria is a human autosomal recessive disorder of organic acid metabolism resulting from a functional defect in the activity of the enzyme methylmalonyl-CoA mutase. Based upon the homology of the human mutase locus with the mouse locus, we have chosen to disrupt the mouse mutase locus within the critical CoA binding domain using gene-targeting techniques to create a mouse model of methylmalonic aciduria. The phenotype of homozygous knock-out mice (mut0) is one of early neonatal lethality. Mice appear phenotypically normal at birth and are indistinguishable from littermates. By 15 h of age, they develop reduced movement and suckle less. This is followed by the development of abnormal breathing, and all of the mice with a null phenotype die by 24 h of age. Urinary levels of methylmalonic and methylcitric acids are grossly increased. Measurement of acylcarnitines in blood shows elevation of propionylcarnitine with no change in the levels of methylmalonyl-CoA. This is the first mouse model that recapitulates the key phenotypic features of mut0 methylmalonic aciduria.

Methylmalonic aciduria (MMA, 1 MIM 251000) is an autosomal recessive inborn error of organic acid metabolism. The true incidence of the disorder has been difficult to determine, and variable figures have been reported. A recent review of newborn screening data indicates an incidence of isolated MMA (mutase and cobalamin A/B) in the order of 1 in 140,000 in Australia (1). The condition results from a functional defect in the enzyme methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) either due to a defect in the mutase (mut) gene itself (designated mut  or mut ) or from a defect in the metabolism of the cofactor adenosylcobalamine. MCM is a nuclear-encoded mitochondrial enzyme that catalyzes the conversion of L-methylmalonyl-CoA to succinyl-CoA, which then enters the citric acid cycle. L-Methylmalonyl-CoA is predominantly derived from the catabolism of branched chain amino acids in the diet, odd chain fatty acids via propionyl-CoA, and propionate synthesized from gut flora.

Following normal pregnancy and delivery-affected individuals with the mut 0 form typically present in the newborn period with overwhelming illness consisting of acidosis, vomiting, poor feeding, hypotonia, and lethargy. Untreated, there is progression to coma and death. The mut0 form may have a less severe phenotype. Aggressive supportive therapy results in survival. However, any intercurrent illness or “metabolic stress” may lead to metabolic instability characterized by repeat recurrent life-threatening episodes of metabolic decompensation.

Currently, the mainstay of treatment for MMA is strict dietary restriction and drug manipulation. The aim of such treatment is to control methylmalonate (MM) and propionate production by limiting dietary intake of precursors and endogenous production of substrate by catabolism. Despite such treatment, morbidity and mortality remain high (2). There are many long term complications such as poor appetite and growth, pancreatitis, cardiomyopathy, and metabolic stroke resulting in permanent neurological damage. Individuals that survive infancy develop renal failure later in childhood and adolescence (3). The underlying pathophysiology leading to these complications is poorly understood.

Liver and combined liver/kidney transplantsations have been attempted with the aim of replacing the deficient enzyme activity, thus preventing the need for diet and avoiding the long term complications. The outcome of such treatment has been successful in allowing patients to relax the dietary restrictions and preventing acute metabolic decompensations (4). However, the natural history of the renal failure and propensity to metabolic stroke remain unclear at this stage (5). Furthermore, liver transplantation is restricted because of the risks associated with it as well as the limitation of suitable donors. A better understanding of the pathophysiology of the disease is therefore needed to enable the development of an effective therapy for this disorder. However, progress has been limited by the lack of animal models for this disorder.

The human mutase gene is ubiquitously expressed and encodes for a 750 amino acid precursor protein including a 32 amino acid mitochondrial leader sequence (6, 7). The leader
sequence is cleaved within the mitochondria, and a mature enzyme is formed. Three functional domains have been proposed as follows: (i) the C terminus (residues 578–750), which comprises the covalin binding domain; (ii) the (βα)₈ barrel (residues 87–416) to which the CoA of methylmalonyl-CoA binds; and (iii) the N terminus with a region involved in the dimerization of the two MCM monomers (residues 32–87).

Cloning of the mouse locus revealed a 94% homology to the human amino acid sequence (8). Furthermore, the mouse cDNA was shown to complement patient cell lines, thus confirming its homology at a functional level. To develop a mouse model for mut⁺⁺ MMA, we have disrupted the mouse mutase locus by gene targeting. We have chosen to disrupt the mouse locus within the CoA binding domain by replacement of exon 3 with an antibiotic selection marker by homologous recombination. This is the first mouse model to be developed for the mut⁻⁻⁻ form of MMA disease. Here we describe the observed phenotype and the biochemical characterization of these mice.

**EXPERIMENTAL PROCEDURES**

**Construction of Targeting Cassette** —The primers 2aF, 5'-TGAGGATATTCATGCTCAG-3', and 2aR, 5'-CTGCACTACTGAGTGTCG-3', were designed to amplify a 349-bp product encompassing mouse mutase exon 2. Similarly, the primers 13F, 5'-ATGGATCCTCAGCCAACAC-3', and 13R, 5'-TGTACAGATCAGCGTGTTTC-3', were designed to amplify a 280-bp product encompassing mouse exon 13. The target vector was constructed by subcloning an 11.2-kb NheI/BamHI restriction fragment containing exons 2 and 6 of the mouse mutase locus. The targeting vector was linearized by restriction digestion with EcoRV. Settings were 0.8 kV, 3

**Southern blot analysis** of genomic DNA from homozygous (Mut⁻⁻⁻) knock-out mice. Shown is RT-PCR of total RNA extracted from liver of (lane 1) wild type, (lane 2) knock-out allele, (lane 3) mouse heterozygous for the exon 3 deletion (Mut⁺⁺⁻⁻), (lane 4) neonate mouse homozygous for the exon 3 deletion (Mut⁻⁻⁻⁻), giving a PCR product only with the neomycin/kanamycin primers. B, Southern blot analysis of genomic DNA from homozygous (Mut⁻⁻⁻⁻) (lane 1), heterozygous (Mut⁻⁻⁻⁻⁻⁻) (lane 2) and wild type (Mut⁺⁻⁻⁻) (lane 3) mice after EcoRI/PstI restriction digestion and probing with an exon 2 PCR product. The wild type locus corresponds to the 3204-bp band, whereas the knock-out allele corresponds to the 5712-bp band. C, as for B after digestion with SacI and probing with an exon 6 PCR product, giving a 8567-bp band with the wild type allele and a 13,782-bp band with the knock-out allele. D, analysis of mRNA expression in knock-out mice. Shown is RT-PCR of total RNA extracted from liver of (lane 1) wild type, heterozygous (lane 2), and homozygous (lane 3) knock-out animals using primers amplifying between exons 2 and 6 of the mouse mutase locus together with control primers for the mouse FrdA locus.

containing the selection cassette in the reverse orientation were used for targeting into embryonic stem (ES) cells. This construct when homologously recombined into the mouse mutase locus should cause deletion of exon 3 (Fig. 1C). If the selection cassette is spliced out by splicing between exons 2 and 4, it should produce a frameshift with premature protein truncation and total loss of enzyme function.

**Generation and Microinjection of Targeted Embryonic Stem Cells** —Mice ES cells from a W9.5 line were used for transfection. Cells (5 × 10⁴) were electroporated with 30 µg of pBR_mutB DNA, which had been linearized by restriction digestion with EcoRV. Settings were 0.8 kV, 3 microfarads, and maximum Ω using a Bio-Rad gene pulser (Bio-Rad). Transfected ES cells were grown on STO Neo⁺ feeder cells using standard ES medium containing leukemia inhibitory factor (Chemicon Inter-
national, Temecula, CA) and β-mercaptoethanol. G418 (Invitrogen) anti-
biosis at a concentration of 200 μg/ml was added after 24 h and continued for
7 days. Resistant colonies were picked from day 6 to day 11. Cell lines were
established and stored at ~70 °C. An aliquot of each was
seeded onto gelatin plates and grown to confluence. DNA was extracted and
used for PCR screening to detect correct recombinants. Correctly
targeted cell lines were initially determined by PCR using the primers
sF, 5'-CAGGCTCATCCCTACCATGT-3' and rF, 5'-TTCCTGTGAT-
TCATCGACTGT-3', to give a product of 3 kb (Fig. 1C).

Targeted cell lines were karyotyped and confirmed by fluorescent in situ
hybridization and Southern blot analysis to contain a single tar-
getting event (data not shown). Standard methods were used to micro-
inject the targeted ES cell line into C57BL/6 blastocysts (10). These
were transferred into pseudopregnant Swiss HSDoIa-recipient mice.
Chimeric mice were detected by coat color and mated with C57BL/6
mice to produce heterozygotes. Heterozygous progeny were interbred to
produce homozygous knock-out mice.

Genotyping and Southern Analysis of Mice—DNA was extracted from
mice tail by standard methods and used for PCR genotyping (8).
Primers used for screening were as follows: neoF, 5'-CAACAGA-
CAATCCGCGTCTC-3'; neoR, 5'-GTCAGGAGAGATCCTCGC-3'
(485 bp); expF, 5'-GGGATACCTACCATGT ATAC-3' and expR, 5'-
ACAGTGCAATATAGAAGCAC-3' (1243 bp). Southern blot analysis
was performed on genomic DNA restriction digested with EcoRI and
PstI and probed with a 250-bp exon 2 fragment to confirm correct
targeting of the 5' end of the construct. Correct targeting was confirmed at the
3' end by SacI digestion and probing with a 200-bp exon 6 PCR
probe (Fig. 1C).

RT-PCR Analysis—Total RNA was extracted from liver using the
RNAeasy kit (Qiagen, Hilden, Germany) in accordance with the manu-
facturer's instructions. First strand synthesis was carried out with
Superscript™ RNase H reverse transcriptase kit (Invitrogen) using a
random primer. PCR was carried out using primers 2F, 5'-TTTTCTGGAT-
TCATCGACTGT-3', to give a product of 3 kb (Fig. 1B).

For the measurement of acylcarnitines by tandem mass spectrome-
try, blood was collected from newborn mice at various time points after birth.
Organic acids were extracted with ethyl acetate, converted to trimethylsilyl deri-
atives, and analyzed by gas chromatography-mass spectrometry using
an Agilent 5973 system (Palo Alto, CA) (12). Selected ion monitoring at
287 m/z (methyl citric acid) and 247 m/z (methylmalonic acid)
was performed.

For measurement of urine MM levels by electrospray tandem mass spectrometry, a 2-μl volume of urine was mixed with 50 μl of 100 μM
3H2-MM (MSD Isotopes, Montreal, Canada) and 220 μl of 50% aceton-
itrile/water (v:v). A 30-pl aliquot of this mixture was analyzed by flow injection
up to 50 μl/min of 50% acetonitrile/water (v:v) infused into the
ion source of a Quattro LC tandem mass spectrometer (Micromass,
Manchester, United Kingdom) with multiple reaction monitoring for
117 > 73 m/z (MM) and 120 > 76 m/z (3H2-MM).

For the measurement of acylcarnitines by tandem mass spectrome-
try, blood was collected from newborn mice at various time points and spotted onto absorbent cotton fiber paper (Guthrie) cards. After drying,
3-mm spots were excised from the cards, extracted with methanol, and
butylated in microtiter plates according to standard methods (13). The
same flow analysis parameters as above were used for the measure-
ment of acylcarnitines, including the levels of C4, C5, and C6 carnitines.
Multiple reaction monitoring transitions (M + H > 85 m/z) were used to
measure the acylcarnitines.

Histopathology—Tissue samples were collected from culled mouse
pups. Kidney, liver, and the intact brain were dissected and placed in
10% formalin. Coronal sections were taken from each organ of wild type
mice, heterozygous mice, and homozygote knock-out mice at various
times after birth and stained with hematoxylin and eosin (H&E) or
periodic acid Schiff reagent.

RESULTS

The targeting construct, pBR_mutB (Fig. 1B), was designed
to replace exon 3 of the mouse mutase locus with a neomycin/
kanamycin selection cassette, running in the reverse orientation,
flanked by loxP sites. The exon 3 region is critical for CoA
binding, and its disruption should result in a loss of function.
Furthermore, even if the selection cassette was deleted by
alternative splicing between exons 2 and 4, the deletion should
lead to premature termination. A low targeting frequency in
the order of 1 in 1000 was obtained between the targeting
construct and homologous sequences in mouse ES cells. One
correctly targeted ES cell line was identified by PCR screening,
Southern blot analysis, and fluorescent in situ hybridization
analysis (data not shown). However, the first chimeric animals
generated after injection of this cell line into C57BL/6 blasto-
cysts were inexplicably lost over the first weeks after birth
along with non-chimeric animals. No cause could be estab-
lished for the loss of these animals. The injection was repeated
and resulted in two germ line chimeras. These mice were
crossed with C57BL/6 to produce heterozygous progeny and
establish a healthy colony of heterozygous knock-out animals.
Heterozygous knock-out animals are phenotypically normal
and have normal growth and fertility. Heterozygous mice were
intercrossed to produce homozygous knock-out mice. Among 76 off-
spring from 12 littersmates from such crosses, we identified by

FIG. 3. Analysis of urine metabolites in homozygous knock-out
animals. A, gas chromatography-mass spectrometry—ion-mon-
itoring chromatograms showing increase in MM and methylcitric (MC)
acid levels in homozygous knock-out mice (KO) compared with wild type
control mice (C). Methylcitric acid gives two peaks due to the formation
of diastereoisomers. B, methylmalonic acid was measured by tandem
mass spectrometry in urine samples of homozygous knock-out animals
at various time points after birth. Accumulation of urinary MM in
homozygous knock-out mice with time was observed.

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PCR screening 17 animals that had the homozygous knock-out genotype (Fig. 2A), a result consistent with the expected Mendelian frequency. Southern blot analysis for the 5’ and 3’ ends of the targeting cassette gave the expected size fragments (Fig. 2, B and C), also confirming homozygosity for the knock-out allele in affected neonates.

Homozygous null mice were born normally and were indistinguishable from their normal littermates at birth. They were of comparable birth weight and initially were active, vigorous, and suckled normally. However, the neonates developed intermittent gasping respirations and stopped suckling (observation of pups behavior along with evidence of reduced/absent milk spots). Within a few hours, homozygous pups developed intermittent gasping respirations and had to be humanely sacrificed. Homozygous null mice did not survive beyond 24 h of age.

Homozygous null mice were born normally and were indistinguishable from their normal littermates at birth. They were of comparable birth weight and initially were active, vigorous, and suckled normally as evidenced by the observation of large milk spots in the stomach shortly after birth. By 15–18 h of age, homozygous null mice gradually became less active and stopped suckling (observation of pups behavior along with evidence of reduced/absent milk spots). Within a few hours, homozygous pups developed intermittent gasping respirations and had to be humanely sacrificed. Homozygous null mice did not survive beyond 24 h of age.

The mutase expression was examined in total RNA extracted from liver tissue of wild type, heterozygous, and homozygous knock-out mice. RT-PCR was performed using primers flanking the targeting cassette. No product was observed in homozygous mice. Control primers from the widely expressed Frda locus were used to confirm integrity of the RNA (Fig. 2D).

Biochemical Characterization—Enzyme activity was assessed indirectly using [14C]propionate incorporation in cultured fibroblasts. Measurements were performed in duplicate, giving incorporation of 653 pmol propionate/mg protein/18 h in wild type mice compared with 39 pmol propionate/mg protein/18 h in homozygous knock-out mice. The residual enzyme activity detected in homozygous knock-out animals corresponded to only approximately 6% of the activity relative to wild type mice.

MM and methylcitric acid were grossly increased in urine as assessed by gas chromatography-mass spectrometry (Fig. 3A). Measurement of urinary MMA levels collected at various time points post-delivery and measured using tandem mass spectrometry identified grossly elevated MM levels immediately after birth with a sequential increase over time (Fig. 3B). There was no significant difference between levels in heterozygotes and wild type mice, although the method used may not have adequate sensitivity to detect small increases in heterozygotes.

Analysis of urine by dipstick showed a urinary pH of 6 with none of the urine samples tested (n = 15), showing evidence of ketonuria. This included testing of urine samples immediately prior to death and with greatly elevated MM levels.

Analysis of acylcarnitines identified the mean of propionylcarnitine (C3) levels to be approximately 6 times greater than mean levels in heterozygous and wild type mice (Table I). There was no significant difference between knock-out and wild type mice for the other acylcarnitines. A comparison of the ratio of propionylcarnitine to free carnitine (C3:C0) and to acetylcarnitine (C3:C2) showed that these ratios were approximately 7-fold higher in homozygous mice when compared with unaffected controls (Table I). These ratios have similarly been shown to be increased in human MMA subjects (14).

Histology of the kidney showed no identifiable abnormality in homozygous mice. Sections were taken through five regions of the brain, and no gross histological abnormalities were detected on H&E stain (data not shown). Examination of liver samples taken within 12 h of birth was normal in homozygous knock-out animals in contrast to samples collected at around 20 h of age, which showed evidence of moderate fatty change in liver parenchyma (Fig. 4).

### DISCUSSION

Mice homozygous for the knock-out allele demonstrate a phenotype similar to that of the human mut−/− MMA. As frequently occurs with the human MMA disorder, they are born normally and develop disease in the newborn period once they are removed from the maternal circulation and commence ingestion of protein. The maternal circulation in this model also appears to be protective of the fetus, at least to the extent that mice develop normally in utero and are born healthy. Nevertheless, levels of MMA in the urine are already significantly elevated even with the first sample collected straight after birth. This suggests the maternal circulation does not completely “detoxify” the fetus. This finding is in agreement with the observed elevation of odd-numbered long-chain fatty acids in fetuses in disorders of propionate metabolism, reflecting the ongoing in utero elevation of propionyl-CoA despite the maternal circulation (15). The significance of this to the health of the developing fetus is unclear at this stage.

Studies of RNA by RT-PCR in these mice failed to show any mutase product in homozygotes. The targeting cassette was designed such that if the antibiotic marker was alternatively spliced the resulting product would result in a frameshift with the capability to detoxify the fetus. This finding is in agreement with the observed elevation of odd-numbered long-chain fatty acids in fetuses in disorders of propionate metabolism, reflecting the ongoing in utero elevation of propionyl-CoA despite the maternal circulation (15). The significance of this to the health of the developing fetus is unclear at this stage.

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Biochemical analyses of urine from homozygous mice demonstrated the characteristic biochemical changes observed in MMA. They have elevated urinary levels of methylmalonic and methylcitric acids and an elevation in blood of C3 or propionylcarnitine in the range observed with human disease (12, 14). The C3:C2 and C3:C0 ratios, which are used as another indicator for the identification of the organic acid disorders MMA and propionic acidemia (PA) in newborn-screening programs, are also elevated in our animal model.

<table>
<thead>
<tr>
<th>Genotype/phenotype</th>
<th>C0</th>
<th>C2:C0</th>
<th>C3:C2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous knock-out (n = 5)</td>
<td>18.66 ± 6.9</td>
<td>0.57 ± 0.26</td>
<td>0.48 ± 0.18</td>
</tr>
<tr>
<td>Control (n = 4)</td>
<td>2.9 ± 0.44</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.02</td>
</tr>
</tbody>
</table>
Massive ketosis with hyperglycinuria was first reported in the early 1960s in an infant suffering from a disorder in the pathway of propionate metabolism that was later confirmed to be PA (16). Since that time, there have been many reports of individuals with PA or MMA describing them as disorders with massive ketoacidosis. Therefore, it is interesting that our mouse model does not have evidence of ketosis on dipstick testing of the urine of the homozygous knock-out mouse as early as 10 h of age (17). In this context, it is interesting that a study on two children with MMA reported that they were not particularly ketogenic during illnesses (18). However, one patient demonstrated an elevation of ketones following loading with protein, l-valine, propionate, and l-leucine. It was postulated that MM-CoA may be toxic to citrate synthase, leading to an increase in acetyl-CoA used for ketone body production. This could be exacerbated by hypoglycemia, an observed secondary affect of elevated MM-CoA, leading to utilization of fat and therefore further ketone body production. In another infant with B12-responsive MMA, a rise in ketones prior to the increase in methylmalonic acid was observed, which did not occur until 12–24 h into the illness (19). This finding has prompted the clinical practice of measuring urinary ketones in MMA patients with the onset of intercurrent infections to detect impending instability in MMA. A review of the literature for other case reports of MMA indicates that the occurrence of ketosis in MMA is variable with a significant number of patients not developing ketosis. Although individuals with propionic acidemia may have a greater propensity to develop ketosis, a patient has also been described in whom this was not a consistent finding (20), suggesting that a more rigorous evaluation of the occurrence and triggers for ketosis in these disorders may be warranted.

Histopathology on the liver shows that our homozygous knock-out model rapidly develop fatty changes with increasing levels of methylmalonic acid. Similar findings were also reported in homozygous knock-out mice for PA (17). Variable hepatomegaly has been described in a number of cases of MMA, whereas fatty change has also been reported as a histological finding (21). This is probably because of a secondary effect of elevated propionyl-CoA or other metabolites on fatty acid synthesis (22). The MMA knock-out model described in this report is the first mouse model for this disease. The accurate recapitulation of the main phenotypic features of the disease should make this mouse model an invaluable tool for further investigations into the pathophysiology of MMA, particularly in relation to the neurological complications of this disease as well as for the development of much needed novel therapies for this disorder.

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