Liver-Specific Deletion of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Causes Hepatic Steatosis and Death

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Objective—3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) catalyzes the rate-limiting step in cholesterol biosynthesis and has proven to be an effective target of lipid-lowering drugs, statins. The aim of this study was to understand the role of hepatic HMGCR in vivo.

Methods and Results—To disrupt the HMGCR gene in liver, we generated mice homozygous for a floxed HMGCR allele and heterozygous for a transgene encoding Cre recombinase under the control of the albumin promoter (liver-specific HMGCR knockout mice). Ninety-six percent of male and 71% of female mice died by 6 weeks of age, probably as a result of liver failure or hypoglycemia. At 5 weeks of age, liver-specific HMGCR knockout mice showed severe hepatic steatosis with apoptotic cells, hypercholesterolemia, and hypoglycemia. The hepatic steatosis and death were completely reversed by providing the animals with mevalonate, indicating its essential role in normal liver function. There was a modest decrease in hepatic cholesterol synthesis in liver-specific HMGCR knockout mice. Instead, they showed a robust increase in the fatty acid synthesis, independent of sterol regulatory element binding protein-1c.

Conclusion—Hepatocyte HMGCR is essential for the survival of mice, and its abrogation elicits hepatic steatosis with jaundice and hypoglycemia. (Arterioscler Thromb Vasc Biol. 2012;32:1824-1831.)

Key Words: cholesterol [ liver [ 3-hydroxy-3-methylglutaryl coenzyme A [ fatty acids [ knockout mouse

The mevalonate pathway produces isoprenoids that are essential for the diverse cellular functions ranging from cholesterol synthesis to growth control. The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) (EC 1.1.1.34), which catalyzes the conversion of HMG-coenzyme A to mevalonate, is the rate-limiting enzyme in the mevalonate pathway.1 Mammalian HMGCR is an integral high-mannose glycoprotein of the endoplasmic reticulum (ER).2 Structurally, it is divided into 2 major domains: a C-terminal cytosol-facing domain that tetramerizes to form the active site and an N-terminal hydrophobic region that spans the ER membrane 8x and bears a single N-glycan. This membrane region is dispensable for the enzymatic activity but necessary for the metabolically controlled stability of the enzyme and sufficient to cause sterol-accelerated degradation of heterologous proteins. Within this region, transmembrane spans 2 through 6 bear significant sequence homology to the corresponding transmembrane spans of sterol regulatory element binding protein (SREBP) cleavage-activating protein (SCAP).3

To ensure a steady supply of mevalonate, the nonsterol and sterol end products of the mevalonate metabolism exert feedback regulatory effects on the activity of this enzyme through multivalent mechanisms, including inhibition of the transcription of its RNA, blocking of translation, and acceleration of the protein’s degradation by a mechanism called ER-associated degradation, thereby regulating the amount of protein over a several hundred-fold range. ER-associated degradation of HMGCR requires the binding of Insig-1 to the sterol-sensing domain.4 Despite the critical role of HMGCR in cholesterol biosynthesis, little is known about its relevance to diseases. Only recently, HMGCR has been identified as a determinant of plasma cholesterol levels.5

Inhibitors of HMGCR, statins, are potent cholesterol-lowering agents that have been widely used to prevent the occurrence of coronary heart disease and other atherosclerotic diseases.6 The atheroprotective properties of statins are primarily because of the potent low-density lipoprotein (LDL)-cholesterol–lowering effect.7 Statins have also been reported to exert cholesterol-independent, or so-called pleiotropic, effects that involve improving endothelial function and decreasing oxidative stress and vascular inflammation.8 The benefits of statins extend beyond cardiovascular diseases and include a reduction in the risk of dementia, Alzheimer disease, ischemic stroke, osteoporosis, tumor growth, and viral infection. Most of these pleiotropic effects are mediated by an ability to block the synthesis of nonsterol isoprenoid intermediates. Statins have toxic effects in only a limited number of patients and are generally considered safe.
To establish an animal model for investigating the mechanisms behind the effects, including toxicity of statins, we have generated HMGCR knockout (KO) mice lacking the enzyme throughout their bodies. These mice die in a relatively early stage of embryonic development (ie, before E8.5). Because mice lacking squalene synthase, the first committed enzyme in the sterol pathway, die in the embryonic stage with apparent anomalies of the development of the central nervous system, we took advantage of the tissue-specific gene targeting using the Cre-loxP system to generate mice lacking HMGCR in a liver-specific manner.

Materials and Methods
Liver-specific HMGCRKO (L-HMGCRKO) mice were generated by cross-breeding heterozygous floxed HMGCR (referred to as HMGCR<sup>f/f</sup>; f denotes floxed) mice with transgenic mice expressing Cre recombinase under the control of albumin gene promoter (Alb-Cre). All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at Jichi Medical University. For detailed protocols for the generation of HMGCRf/f (fHMGCR) mice, we used fHMGCR mice as a control. To determine whether the expression of the HMGCR gene was abrogated in the liver, we performed a Southern blot (Figure 1A), Northern blot (Figure 1B), and real-time polymerase chain reaction (Figure 1C). The Southern blot analysis shows that only the 6.3-kb wild-type allele and 7.7-kb floxed allele were observed in the DNA from liver in wild-type and fHMGCR mice, respectively. In L-HMGCRKO mice at 3 weeks, the liver contained predominantly the 12.0-kb KO allele (70% based on PhosphorImager analysis [BAS 200, Fujifilm, Tokyo, Japan]) and some residual floxed allele (30%). The mRNA expression of HMGCR in the liver of L-HMGCRKO mice was decreased to 30% of the fHMGCR mice at 3 weeks of age and to <10% at 4 and 5 weeks upon Northern blot analysis. The time-dependent abolition of the mRNA expression of HMGCR was also confirmed by real-time polymerase chain reaction. Based on this method, the mRNA levels were decreased to only 2% of the fHMGCR mice at 4 and 5 weeks, although there were no significant differences in other organs (Figure IIA in the online-only Data Supplement). Immunoblot analysis showed that the amount of HMGCR protein in the liver of L-HMGCRKO mice was 15% of that in fHMGCR mice (Figure 1D). Despite the profound reduction in HMGCR at both the mRNA and protein levels, the HMGCR activity in the liver of L-HMGCRKO mice was decreased by only 44% compared with that in fHMGCR mice (Figure 1E). To determine whether HMGCR is specifically abrogated in the liver parenchymal cells of L-HMGCRKO mice, we separated parenchymal and nonparenchymal cells from the liver at 3 weeks of age by density-gradient centrifugation after liver perfusion with collagenase. The mRNA expression of HMGCR in the parenchymal cells of L-HMGCRKO mice was decreased by 70.6% compared with that in fHMGCR mice, whereas the mRNA expression of HMGCR in the nonparenchymal cells was not different between the fHMGCR and L-HMGCRKO mice at 3 weeks of age (Figure IIC in the online-only Data Supplement).
Liver Weight and Plasma Parameters of Control and L-HMGCRKO Mice

<table>
<thead>
<tr>
<th>Age (wks)</th>
<th>Genotype</th>
<th>fhMGCR (n=5)</th>
<th>L-HMGCRKO (n=5)</th>
<th>fhMGCR (n=9)</th>
<th>L-HMGCRKO (n=7)</th>
<th>fhMGCR (n=6)</th>
<th>L-HMGCRKO (n=5)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>3</td>
<td>10.6±1.3</td>
<td>11.7±1.5</td>
<td>17.2±1.3</td>
<td>15.5±0.8*</td>
<td>21.7±2.0</td>
<td>13.5±0.8***</td>
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<tr>
<td>Liver weight (g)</td>
<td></td>
<td>0.47±0.11</td>
<td>0.51±0.11</td>
<td>0.78±0.14</td>
<td>0.92±0.13</td>
<td>1.08±0.11</td>
<td>1.17±0.28</td>
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<tr>
<td>Liver/body weight (%)</td>
<td></td>
<td>4.4±0.4</td>
<td>4.2±0.4</td>
<td>4.4±0.4</td>
<td>6.1±0.8*</td>
<td>5.0±0.12</td>
<td>8.7±2.1**</td>
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<td>Total cholesterol (mg/dL)</td>
<td>3</td>
<td>63.6±9.1</td>
<td>47.1±1.0**</td>
<td>66.5±11.1</td>
<td>46.7±13.2**</td>
<td>64.6±6.8</td>
<td>282.6±72.6***</td>
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<td>Free cholesterol (mg/dL)</td>
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<td>26.2±4.0</td>
<td>20.1±0.5*</td>
<td>33.3±5.5</td>
<td>24.6±13.5</td>
<td>33.1±2.7</td>
<td>263.0±72.6***</td>
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<td>Cholesterol ester (mg/dL)</td>
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<td>37.4±5.5</td>
<td>20.7±1.4*</td>
<td>33.1±5.7</td>
<td>22.1±4.6*</td>
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<td>Triglyceride (mg/dL)</td>
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<td>51.1±9.9</td>
<td>78.5±25.4</td>
<td>24.9±6.9***</td>
<td>92.5±31.1</td>
<td>27.7±9.4**</td>
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<td>Free fatty acids (mmol/L)</td>
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<td>0.51±0.19</td>
<td>0.55±0.09</td>
<td>1.70±0.92*</td>
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<td>Phospholipids (mg/dL)</td>
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<td>132.2±20.2</td>
<td>106±2.7*</td>
<td>143.5±22.8</td>
<td>113.7±31.7</td>
<td>150.9±12.3</td>
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<td>Aspartate aminotransferase (IU/L)</td>
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<td>76.1±10.2</td>
<td>95.7±45.5</td>
<td>92.7±39.3</td>
<td>343.1±602.8</td>
<td>55.9±18.6</td>
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<td>Alanine transaminase (IU/L)</td>
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<td>35.1±12.1</td>
<td>61.9±46.7</td>
<td>20.7±17.5</td>
<td>54.2±101.7</td>
<td>13.1±4.6</td>
<td>878.8±357.6**</td>
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<td>Total bilirubin (mg/dL)</td>
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<td>0.30±0.08</td>
<td>0.36±0.29</td>
<td>0.33±0.19</td>
<td>3.25±1.69</td>
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<td>Blood glucose (mg/dL)</td>
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<td>110.6±16.7</td>
<td>123.0±15.5</td>
<td>115.9±13.7</td>
<td>99.6±16.7</td>
<td>135.7±9.4</td>
<td>48.3±23.3*</td>
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</tbody>
</table>

Blood samples were taken from male mice fed a normal chow diet ad libitum before the study. Each value represents the mean±SD. Significant differences compared with control mice: *P<0.05, **P<0.01, and ***P<0.001. HMGCR indicates 3-hydroxy-3-methylglutaryl-coenzyme A reductase; L-HMGCRKO, liver-specific HMGCR knockout mice; fhMGCR, floxed HMGCR.

Online-only Data Supplement. HMGCR activity in the parenchymal cells of L-HMGCRKO mice was decreased by only 47% compared with that in fhMGCR mice (Figure IID in the online-only Data Supplement). The relative decreases in the mRNA and activity of HMGCR in the parenchymal cells were similar to those observed in the whole liver of L-HMGCRKO mice (Figure 1C and E), supporting that HMGCR gene was specifically abrogated in the parenchymal cells of the liver and that there was no compensatory upregulation of HMGCR in the nonparenchymal cells of the L-HMGCRKO mice.

L-HMGCRKO mice were born at a rate in accordance with the rule of Mendelian inheritance. However, 96% of males died with a median survival time of 35 days, whereas 71% of females died with a median survival time of 36 days (Figure 1F), and the rest of them survived until 12 months of age. Therefore, the survival rate of females was significantly higher than males (P=0.025). The mRNA expression levels of HMGCR in the liver from the surviving HMGCRKO mice were as high as those from fhMGCR mice (Figure IIB in the online-only Data Supplement). Next, we compared body weight, liver weight, plasma lipid levels, liver functions, and blood glucose levels between fhMGCR and L-HMGCRKO mice (Table). Although the body weight of L-HMGCRKO mice was not different from that of fhMGCR mice at 3 weeks of age, it decreased by 10% at 4 weeks and by 38% at 5 weeks of age. Liver weight did not differ between the 2 groups. However, the ratio of liver weight to body weight was increased by 39% and 74% at 4 and 5 weeks of age, respectively. The livers of L-HMGCRKO mice were enlarged, paler, and whiter than those of fhMGCR mice at 5 weeks of age (Figure 2A and 2B). Consistent with the macroscopic abnormalities of the liver, plasma levels of aspartate aminotransferase, alanine transaminase, and bilirubin were markedly increased in the L-HMGCRKO mice compared with fhMGCR mice at 5 weeks of age. Total cholesterol levels in the plasma were decreased in the L-HMGCRKO mice by 26% at 3 weeks of age (Table). In parallel, plasma concentrations of LDL cholesterol and apolipoprotein (apo) B-100 protein were decreased (Figure IIIA and IIIE in the online-only Data Supplement). Despite the liver failure of L-HMGCRKO mice at 5 weeks of age, the plasma levels of cholesterol, free cholesterol, and phospholipids were increased 4.3-, 7.9-, and 4.0-fold, respectively. On the other hand, plasma cholesterol ester levels were decreased by 38% (Table). Therefore, calculated ratios of free cholesterol to cholesterol ester were increased 13-fold. Most of the increased cholesterol was distributed in the size of very low-density lipoprotein through LDL (Figure IIIC in the online-only Data Supplement). Although apoB-100 levels were decreased, apoB-48 levels were substantially increased in the plasma of L-HMGCRKO mice at 5 weeks of age (Figure IIIG in the online-only Data Supplement). These findings together with the results of agar gel electrophoresis of the plasma (Figure III-I in the online-only Data Supplement) strongly indicate that both lipoprotein X and apoB-48–containing lipoproteins were increased in the L-HMGCRKO mice. Hepatic LDL receptor protein levels were not altered between fhMGCR and L-HMGCRKO mice at 3 and 5 weeks of age (Figure IIIF and IIIH in the online-only Data Supplement), despite increased hepatic LDL receptor mRNA levels (Figure 4A). Plasma lipoprotein changes in L-HMGCRKO mice may not be influenced by LDL receptor protein levels. Plasma free fatty acid levels were also increased 3.1-fold at 5 weeks. In contrast, plasma triglyceride levels were decreased by 69% and 70% at 4 and 5 weeks of age, respectively. It is of note that L-HMRCRKO mice were moderately hypoglycemic at 5 weeks.

To determine the causes of the liver dysfunction associated with hepatomegaly, we performed a microscopic analysis of the liver (Figure 2). Hematoxylin and eosin staining revealed moderate to severe ballooning and uniconcave necrosis of parenchymal cells at 5 weeks (Figure 2C and 2D). Oil-red O staining showed moderate to severe microvesicular steatosis of the parenchymal cells (Figure 2E and 2F). TdT-mediated dUTP-nick end labeling–positive (Figure 2G, 2H, and 2M) and Ki-67–positive parenchymal cells (Figure 2H, 2I, and 2M).
(Figure 2I, 2J, and 2N) were significantly increased 11- and 2.8-fold in the liver of L-HMGCRKO mice compared with that in fHMGCGR mice, respectively. The liver of L-HMGCRKO mice contained increased amounts of type 4 collagen (Figure 2K and 2L). Caspase 3 activity in the liver of L-HMGCRKO mice was also increased 1.8-fold compared with that of fHMGCGR (Figure VA in the online-only Data Supplement). The mRNA levels of C/EBP-homologous protein were also significantly increased (Figure VE in the online-only Data Supplement). However, no significant differences were observed in either caspase 8 activity or H2O2 contents in the liver (Figure VB and VC in the online-only Data Supplement). The pathology of the liver from L-HMGCRKO mice at 4 weeks was milder than that at 5 weeks. The liver of the mice which survived the lethal period still had hepatocyte ballooning (Figure IVD in the online-only Data Supplement).

To determine whether the liver-specific abrogation of HMGCR affected cholesterol metabolism in the liver, we measured the hepatic levels of cholesterol and triglycerides (Figure 3). The hepatic cholesterol content was decreased by only 22% in the L-HMGCRKO mice compared with the fHMGCGR mice (Figure 3A). Unexpectedly, the hepatic contents of triglyceride were increased 2-fold (Figure 3B). These results indicate that the neutral lipids stained with oil-red O in the liver were primarily triglycerides. The reduction in the cholesterol contents of the liver was smaller than expected. We measured cholesterol synthesis in liver slices in culture (Figure 3C). The cholesterol synthesis was reduced by 45%. This reduction paralleled the degree of reduction in HMGCR activity in the liver (Figure 1E). We also measured the amounts of fatty acids synthesized in the liver (Figure 3D). Surprisingly, the synthesis of fatty acids from acetate was markedly increased 17-fold in the liver of L-HMGCRKO mice. Although total amounts of fatty acids were not different between the 2 mice (Figure 3E), the following fatty acid species were significantly increased in the liver of L-HMGCRKO mice: C18:0, C18:1n–9, C20:1n–9, C20:3n–9, and C22:4n–6 (Figure 3F). Given the increased production of fatty acids and triglycerides in the liver, we measured other metabolites of fatty acids such as diglycerides and ceramides. The hepatic levels of diglycerides were increased 1.8-fold (Figure 3G), but those of ceramides were not increased significantly (Figure 3H).

To clarify the mechanisms behind the changes in fatty acid metabolism, we determined the changes in the levels of mRNA expression in various genes involved in cholesterol or fatty acid metabolism in the liver at 4 weeks of age by real-time polymerase chain reaction (Figure 4A). As to the genes involved in cholesterol metabolism, the mRNA levels of SREBP2, LDL receptor, proprotein convertase subtilisin/kexin type 9, and squalene synthase were increased 1.4-, 1.9-, 2.8-, and 3.9-fold, respectively. On the other hand, the mRNA levels of cholesterol 7α-hydroxylase, a rate-limiting enzyme for bile acid synthesis, were decreased by 60%. With regard to the genes involved in fatty acid metabolism, the mRNA levels of fatty acid synthase (FAS), acetyl-CoA carboxylase, and stearoyl-CoA desaturase 2 were increased 2.4-, 1.4-, and 22-fold, respectively. On the other hand, the mRNA expression of SREBP1c and acyl CoA:diacylglycerol acyltransferase 2 was decreased by 80% and 40%, respectively. There were no changes in either the mRNA expression of SREBP1α, a splice variant of SREBP1c, or liver X receptor α (LXRα), an important transcriptional activator of the SREBP1c gene. The mRNA expression of peroxisome proliferator–activated receptor α, a transcription regulator of genes for fatty acid oxidation, was not changed.

HMGCR catalyzes the formation of mevalonate, which is used as a substrate for the synthesis not only of cholesterol but also nonsterols such as isoprenoids, ubiquinone, heme A, and dolichol. To test the notion that death, likely because of severe liver failure, was caused by the alteration of a nonsterol pathway, we evaluated the isoprenylation of small GTP-binding proteins such as H-Ras and Rac1 by measuring the amount of membrane-bound forms (Figure 4B). The ratio of the
membrane-bound form to the cytosolic form of H-Ras or Rac1 was decreased to 0.05 to 0.2 compared with control. These results indicate that the deficiency of HMGR affected the nonsterol pathway more severely than the sterol pathway. We also estimated changes in intracellular signaling molecules (Figure 4C). Akt and c-Met were decreased by 72% and 81%, respectively, whereas phospho-signal transducer and activator of transcription 3 was increased 7.6-fold.

If the lethal phenotype of HMGR deficiency is because of the deficiency of mevalonate, supplementation with mevalonate could theoretically rescue the lethal phenotype. In fact, providing the male mice with water containing mevalonate significantly attenuated the liver dysfunction and hypercholesterolemia (Figure 5A–5C). The pathological abnormalities observed in the male L-HMGCRKO mice were almost normalized by the supplementation with mevalonate (Figure 5D–5G). Consistently, the amounts of the membrane-bound form of H-Ras or Rac1 were restored to normal levels (Figure 5H). Similar improvements were found in female L-HMGCRKO mice (data not shown). No death occurred until 40 days of age in L-HMGCRKO mice supplemented with mevalonate.

Because L-HMGCRKO mice were hypoglycemic (Table), it is possible that hypoglycemia is the direct cause of death. To test this hypothesis, we allowed mice free access to drinking water containing 20% (w/v) glucose. Glucose feeding significantly increased plasma glucose levels (43.5 ± 16.2 mg/dL [n=10] versus 79.5 ± 40.6 mg/dL [n=13] [P=0.01] in males; 36.6 ± 13.3 mg/dL [n=4] versus 65.2 ± 35.6 mg/dL [n=11] [P=0.04] in females). Concurrently, it dramatically improved the mortality of L-HMGCRKO mice (96% versus 13% in males and 71% versus 15% in females) (Figure VI in the online-only Data Supplement).

**Discussion**

Nearly all the male L-HMGCRKO mice died before 40 days of age, whereas 30% of females survived until 12 months of age. Before their death, the mice developed severe hepatic damage with hepatomegaly, steatosis, and hypoglycemia. Thus, we ascribe the lethal effect of the liver-specific deficiency of HMGR
to the hepatic toxicity and hypoglycemia. Unexpectedly, the mice developed hypercholesterolemia before death, although hepatic cholesterol synthesis was significantly reduced. This lethal phenotype was completely reversed by mevalonate or glucose, indicating that mevalonate is essential for the survival of mice and that hypoglycemia is the direct cause of lethality.

The mRNA expression of HMGCR was reduced as early as 3 weeks of age and almost undetectable at 4 weeks. This developmental reduction in the expression is consistent with the developmental induction of the expression of albumin. Other liver-specific KO models using Alb-Cre showed abrogation of the expression of reporter genes at a similar stage. However, the HMGCR activity of the liver was reduced by only 50%. Because the parenchymal hepatocytes from the liver of the L-HMGCRKO mice at 3 weeks of age expressed substantial HMGCR activity, we speculate that most of the activity is derived from upregulated HMGCR expression in a certain subset of hepatocytes that escaped from HMGCR gene inactivation. Indeed, recombination efficiency was only 75% at weaning, and HMGCR protein can be increased 25-fold at the posttranscriptional level.

Figure 4. Relative amounts of various mRNAs and degree of isoprenylation of small GTP-binding proteins and liver regeneration-associated proteins in the livers of control and liver-specific HMGCR knockout (L-HMGCRKO) male mice at 4 weeks of age. A, Total RNA from the livers of mice (n=5 in each group) was subjected to quantitative real-time polymerase chain reaction as described in the online-only Data Supplement. Each value represents the amount of mRNA relative to that in the control mice, which is arbitrarily defined as 1. B, Liver membranous and cytosolic fractions for small GTP-binding proteins or (C) liver tissue lysates for liver regeneration-associated proteins were prepared as described in the online-only Data Supplement (n=3 in each group), and aliquots (30 μg) were subjected to SDS-PAGE and immunoblot analysis. Each value represents mean±SD. Significant differences compared with control mice: *P<0.05 and **P<0.01. LDLR indicates low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; FAS, fatty acid synthase; DGAT, diacylglycerol acyltransferase; SCD, stearoyl-CoA desaturase; LXRα, liver X receptor; PPARα, peroxisome proliferator–activated receptor α; SS, squalene synthase; MTP, microsomal triglyceride transfer protein; CYP7A1, cholesterol 7α-hydroxylase; ACC, acetyl-CoA carboxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; STAT3, signal transducer and activator of transcription 3.

Figure 5. Supplementation of mevalonate. Male mice were given mevalonate in drinking water at a concentration of 5 mmol/L from the age of 28 days to 35 days (n=5 in each group). After supplementation, plasma alanine transaminase (ALT) (A), total bilirubin (B), and total cholesterol (C) levels were measured. The livers were removed from liver-specific HMGCR knockout (L-HMGCRKO) mice not supplemented with mevalonate (−; D and F, respectively) or supplemented with mevalonate (+; E and G, respectively) stained with hematoxylin and eosin or oil-red O. H, Immunoblot analyses for small GTP-binding proteins in membranous and cytosolic fractions of the liver. Each value represents mean±SD. Significant differences compared with control mice: *P<0.001.
Furthermore, there might be a time lag between disappearance of HMGCR protein and cessation of transcription.

Sex dimorphism in lethality is noteworthy. The present results that male L-HMGCRKO mice were more prone to die than females are consistent with the general view that males are more resistant to morbidity and mortality of various liver diseases than males. In addition to protection against liver injury via vasodilating and anti-inflammatory effects, estrogen itself attenuates the development of hepatic steatosis. The mRNA expression levels of HMGCR in the liver of the surviving L-HMGCRKO mice were indistinguishable from those of fHMGCR mice (Figure IIB in the online-only Data Supplement). It is likely that the hepatocytes with a relatively intact HMGCR gene may be extensively regenerated, thereby eventually compensating for the KO in the survivors. In support of this, several Ki-67–positive cells were greatly increased in the L-HMGCRKO mice (Figure 2L and 2N).

Hepatic cholesterol levels were mildly decreased, and the decrease in plasma cholesterol levels was modest in the L-HMGCRKO mice at 3 weeks of age. More surprisingly, the plasma cholesterol levels even increased immediately before death. Extrahaepatic organs with high cholesterol synthesis, such as skin, bowels, and muscles, may be a source of cholesterol in the plasma. The hypercholesterolemia before death appears paradoxical, given the failure of cholesterol synthesis in the hepatocytes. At this stage, the mice develop jaundice. Because the physicochemical characteristics of the accumulated lipoproteins were similar to those of lipoprotein X, cholesterol at least partly account for hypercholesterolemia. Simultaneous accumulation of both lipoprotein X and apoB48-containing particles has also been reported in rats with intrahepatic cholestasis.17

Interestingly, L-HMGCRKO mice showed hepatic steatosis. Because hepatic triglyceride levels were increased 2-fold but hepatic cholesterol ester levels were not increased (data not shown) most of the neutral lipids stained with oil-red O are triglycerides. Supporting this, the synthesis of fatty acids was increased 17-fold. In this context, it is noteworthy that the expression of enzymes for fatty acid synthesis, FAS and stearoyl-CoA desaturase 2, was increased. In normal adults, stearoyl-CoA desaturase 1 is the major enzyme catalyzing desaturation of oleate, whereas stearoyl-CoA desaturase 2 is an isozyme that is transiently expressed in the liver in embryos and neonates and may be involved in lipogenesis at that developmental stage. Indeed, the amounts of polyunsaturated fatty acids were increased in the liver (Figure 3F).

The increase in fatty acids synthesis appeared disproportionately larger than the increase in triglycerides in the liver. In this context, it is of note that the expression of diacylglycerol acyltransferase 1 was not increased and that of diacylglycerol acyltransferase 2 was even decreased. Thus, it is probable that the newly synthesized fatty acids were not used to produce triglycerides. Generally, the enzymes catalyzing fatty acid synthesis are transcriptionally induced by SREBP1c. However, the expression of SREBP1c was reduced 5-fold, suggesting that the increased lipogenesis was not mediated by SREBP1c. Similar SREBP1c-independent lipogenesis is reported in mice overexpressing a constitutively active form of Akt in the liver. Furthermore, the levels of LXRα mRNA, a nuclear receptor that can stimulate SREBP1c gene expression and can also directly stimulate transcription of FAS, were not increased in the livers of L-HMGCRKO mice. We speculate that the lack of cholesterol reduces the supply of oxysterols, physiological ligands of LXR, which transactivates SREBP1c. This notion is supported by the decreased expression of cholesterol 7α-hydroxylase and diacylglycerol acyltransferase 2, also targets of LXR.

In contrast to SREBP1c expression, the expression of SREBP2 was increased, conceivably accounting for the increased expression of its targets: the LDL receptor and squalene synthase. The expression of FAS in the face of significant suppression of SREBP1c or unaltered LXRα suggests a regulatory pathway independent of SREBP1c or LXRα. Recently, a decrease in farnesyl pyrophosphate or farnesol has been shown to induce the expression of FAS independently of SREBP1c.

The liver of the L-HMGCRKO mice contained increased numbers of TdT-mediated dUTP-nick end labeling–positive cells. Consistent with the apoptotic nature of the cell death, the activity of caspase 3, a final executor of apoptosis which cleaves to induce the release of cytochrome c from mitochondria, was increased in the liver of the L-HMGCRKO mice. We have hypothesized several potential mechanisms for apoptosis: accumulation of toxic lipid metabolites and reduction of survival factors. However, we failed to obtain evidence for the involvement of ceramide and survivin in apoptosis.

As predicted, membrane-bound forms of Ras and Rac1 were significantly reduced, conceivably as a result of the defect in their isoprenylation. Several mechanisms linking the defect in isoprenylation to apoptosis have been proposed. For example, Rac1 is reported to protect against apoptosis by stimulating nicotinamide adenine dinucleotide phosphate-oxidase, thereby increasing the levels of reactive oxygen species. However, we failed to detect a decrease in H2O2 levels in the liver.

During the search for the plausible mechanism of the hepatocyte apoptosis, we found a significant increase in the mRNA level of C/EBP-homologous protein, a hallmark of the ER stress response and inducer of apoptosis, in the liver of L-HMCRKO mice. Because hepatic steatosis induces ER stress, it is reasonable to speculate that the increased synthesis of fatty acids causes hepatic lipopapoptosis via increasing the C/EBP-homologous protein. The liver of L-HMGCRKO mice had significantly decreased levels of either p-Akt or c-met protein. Because hepatocyte growth factor exerts pro-survival effects mainly through activating phosphatidylinositol-3 kinase/Akt pathway after binding to its receptor, c-met, defective hepatocyte growth factor signaling may play a salutary role in the induction of hepatocyte apoptosis. The increase in phospho-signal transducer and activator of transcription 3 might be a compensatory response secondary to increased apoptosis, but failed to overcome it. Similar failure to compensate the liver failure by activated signal transducer and activator of transcription 3 was reported in mice deficient in phosphoinositide-dependent protein kinase 1.

These phenotypes of L-HMGCRKO mice should be discussed in relation to other mouse models of genetic disorders of cholesterol metabolism. Thus far, 2 models have been reported to survive the perinatal period, despite reduced cholesterol biosynthesis in the liver. Because the global disruption of SREBP cleavage-activating protein and site 1 protease is embryonic lethal, as is the case for HMGCR, liver-specific KO
mice have been generated. In both the liver-specific SREBP cleavage-activating protein and site 1 protease KO mice, the synthesis of cholesterol as well as fatty acids was significantly reduced in the liver. Neither mouse, however, died of liver toxicity, probably because the reduction in the expression of HMGR was not as severe as in L-HMGCRKO mice.

In conclusion, HMGR is essential for the survival of mice. Despite the defect in hepatic cholesterol biosynthesis in L-HMGCRKO mice, the homeostasis of cholesterol in the liver and plasma is surprisingly well maintained presumably via compensatory changes in the flux of cholesterol and fatty acids. These results might provide insight for understanding the role of cholesterol biosynthetic pathway in the normal function of hepatocytes.

Acknowledgments
We thank Drs Derek LeRoith, Wataru Ogawa, and Masato Kasuga for providing Alb-Cre mice. We also thank Drs Tetsuya Kitamine, Ryuichi Tozawa, Yoshiaki Tamura, Hiroaki Okazaki, Masaki Igarashi, Hitoshi Shimoano, and Nobuhiro Yamada for their help and discussion.

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Disclosures
None.

References
Liver-Specific Deletion of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Causes Hepatic Steatosis and Death
Shuichi Nagashima, Hiroaki Yagyu, Ken Ohashi, Fumiko Tazoe, Manabu Takahashi, Taichi Ohshiro, Tumenbayar Bayasgalan, Kenta Okada, Motohiro Sekiya, Jun-ichi Osuga and Shun Ishibashi

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Supplemental material

Supplemental Methods

*Generation of mice heterozygous for the floxed HMGCR allele*

A conditional targeting vector of a replacement type was produced by inserting a loxP site into a XhoI site in intron 1 and loxP-flanked (floxed) polII-neo-bpA cassette into a BamHI site in intron 4 (Supplemental figure I). The transcriptional orientation of the neo gene was opposite to that of the HMGCR gene. Excision of sequences between the loxP sites by Cre recombinase deletes exons 2 to 4, which includes the initiator methionine and residues encoding the first membrane-bound domain of HMGCR. JH1 ES cells (A gift from Dr. J. Herz) were electroporated with the targeting vector as described\(^1\). Recombinant clones containing a single floxed HMGCR allele were identified by PCR using primers P1

(5’-ACGAAAGGGCCTCGTGATACGCCTA-3’) and P2

(5’-ATGTCTGCAGTCCCAGCACTCAGCT-3’). All targeted clones were confirmed by Southern blot analysis using a cDNA probe containing exons 7-10. The targeted clones were injected into the C57BL/6J blastocysts, yielding two lines of chimeric mice which transmitted the floxed allele through the germ line.

*Generation of liver specific HMGCR knockout mice.*
Mice expressing Cre recombinase under the control of the albumin gene promoter (Alb-Cre) were kindly provided by Dr. D. LeRoith, W. Ogawa and M Kasuga as described in main text were backcrossed with C57BL/6J mice 6 times before interbreeding. HMGCR^{+/f} carrying one copy of the Alb-Cre transgene were interbred with HMGCR^{+/f} littermates lacking Cre to generate liver-specific HMGCR knock-out (HMGCR^{f/f} Alb-Cre; L-HMGCRKO) mice and littermate control [HMGCR^{f/f} (fHMGCR), HMGCR^{+/+};Alb-Cre (CRE), and HMGCR^{+/+} (WT)] mice. Age- and sex-matched littermates were used as the controls. Disruption of the floxed HMGCR allele in the mice was confirmed by Southern blot and Northern blot analyses. Genotyping was performed by PCR using genomic DNA isolated from the tail tip. The primer sequences for the Alb-Cre transgenes were as follows: primer A, 5’GTGGTTAATGATCTACAG 3’; primer B 5’CCTGAACATGTCCATCAG 3’. For floxed HMGCR genotyping, we used as primer A, 5’ GTCGACGTTGAA TCCTCTTGTCAGAC 3’; and primer B, 5’CAAAGCAGACATGAGACTATTC 3’. All mice were group-housed in cages with a 12-hour light/dark cycle and fed CE-2 (Japan CLEA). Unless otherwise stated, they were fed a chow diet ad libitum, and tissues were collected in the early dark phase at a time when HMGCR activity was at its peak of diurnal rhythm$^2$. 

2
Liver parenchymal and non-parenchymal cell isolation.

Liver parenchymal and non-parenchymal cells were isolated using the two-step liver perfusion method as described previously. Because the parenchymal cells from L-HMGCRKO mice after 4 weeks of age were easy to die after perfusion with collagenase, L-HMGCRKO mice at 3 weeks of age were used. In brief, animals were anesthetized with pentobarbital sodium. Then, the abdominal cavity was opened, and the portal vein was cannulated using a 24-G elastic retention needle. The liver was perfused immediately with 20ml of the first perfusate (Ca $^{2+}$ free Hanks' balanced salt solution (HBSS) with 10 mM HEPES, 0.5 mM EGTA and 10 mM glucose, pH 7.4) at a flow rate of 6 ml/min to remove all of the blood. Outflow was performed by cutting the inferior vena cava. The perfusate was changed to 5ml of the second perfusate (HBSS containing 0.5mg/ml type IV collagenase (WAKO), type II trypsin inhibitor (Sigma), 5mM CaCl$_2$ and 10mM HEPES, pH 7.4). The second perfusion was at a same flow rate as the first perfusion. The liver was removed, transferred to a petri dish containing cold Williams E medium (GIBCO) supplemented with 5% fetal calf serum (FCS), and minced gently. Parenchymal and non-parenchymal components were prepared according to Rountree et al. In brief, the cells were centrifuged at 50 x g for 1 minute. The pellet was saved as parenchymal cell-enriched fraction. The supernatant was
centrifuged at 50 x g for 1 minute, and the supernatant centrifugation at 50 x g for 1 minute. The final supernatant was centrifuged at 180 x g for 8 minutes, with the pellet representing the non-parenchymal cell fraction (endothelial cells, Kupffer cells, stellate cells, and biliary cells). Total RNA was extracted from isolated non-parenchymal cells and used for quantitative real-time PCR. The first parenchymal cell-enriched pellet was further purified by density-gradient centrifugation in Percoll⟪. This technique reduces contamination of parenchymal cells by other cell types and by non-viable cells. After incubation in Williams E medium supplemented with 5% FCS for 3 hours, the attached parenchymal cells were subjected to total RNA extraction for quantitative real-time PCR or microsomal protein extraction for HMGCR activity assay.

**Northern blot analysis and quantitative real-time PCR**

Total RNA was prepared from mouse tissues or liver cells using TRIzol (Invitrogen). For the Northern blot analysis, pooled total RNA was subjected to 1% agarose gel electrophoresis in the presence of formalin and was transferred to Hybond N+ membranes (GE healthcare). The membranes were hybridized to 32P-labeled HMGCR cDNA probes containing exons 2-4. Radioactivity was quantified with a BAS 2000 (Fujifilm). For Quantitative real-time PCR, all reactions were done in triplicate and relative amounts of mRNA were calculated using a standard curve or the comparative
CT method with the 7300 Real-Time PCR system (Applied Biosystems) according to the manufacturer’s protocol. Mouse β actin mRNA was used as the invariant control. The primer-probe sets for real-time PCR are listed in supplemental table I.

**Immunoblot analyses of liver cytosolic and membranous fractions.**

To prepare cytosolic and membranous fractions for immunoblot analyses, aliquots of frozen liver (100 mg) were homogenized in 1 ml of buffer (20mM Tris-Cl at pH 7.4, 2mM MgCl2, 0.25M sucrose, 10mM sodium EDTA, and 10-mM sodium EGTA) supplemented with a protease inhibitor cocktail (Sigma). The liver homogenate was centrifuged at 1,000 x g for 5 min at 4°C. The supernatant was removed and used to prepare membranous and cytosolic fractions as described previously. After aliquots of the cytosolic and membranous fraction were removed for measuring protein concentrations with the BCA Kit (Pierce Biotechnology), the remainder from each protein (45 µg) was subjected to SDS-PAGE and immunoblotting. For immunoblot analysis of apolipoprotein B, 2µl of plasma were delipidated and subjected to 3~8% SDS-PAGE. Rabbit polyclonal antibody that detect mouse HMGCR7 was kindly provided by Dr. YK Ho, MS Brown and JL Goldstein. Additional antibodies used include as follows: LDL receptor antibody (R and D Systems), apolipoprotein B (Santa Cruz Biotechnology), H-ras, pan-Akt, pan-Akt (phospho T308), STAT3, STAT3 (phospho
Y705) and c-Met (Abcam), Rac1 (Upstate Biotechnology), GAPDH (Ambion) and transferrin receptor (Zymed Laboratories).

**HMGCR activity assay**

HMGCR activity in the liver microsomal fraction was measured essentially as described previously (Ref. 9 in the main text). Briefly, the microsomal fraction (~50 µg) was incubated in 20 µl of a buffer containing 110 µM DL-[3-\(^{14}\)C] HMG-CoA (20 nCi/nmol), 5 mM NADPH, 10 mM EDTA, 10 mM dithiothreitil, and 100 mM potassium phosphate, pH 7.4, at 37 °C for 60 min. The reaction was terminated by the addition of 10 µl of 2 N HCl and incubation continued for another 30 min at 37 °C to lactonize the mevalonate formed. The \[^{14}\text{C}\] mevalonate was isolated by TLC and measured using \[^{3}\text{H}\] mevalonate as an internal standard. HMGCR activity is expressed as picomoles of \[^{14}\text{C}\] mevalonate formed per minute per mg of protein.

**Lipids and biochemical analysis**

Blood was drawn from the retro-orbital sinus; plasma was separated immediately and stored at −80°C. Blood glucose levels were measured with a FreeStyle blood glucose monitoring system (NIPRO). Concentrations of cholesterol, free cholesterol, cholesterol ester, triglycerides and free fatty acids in plasma and liver were measured as described (Ref.10 in the main text). The liver fatty acid contents were analyzed by
gas-liquid chromatography. Plasma AST, ALT and total bilirubin levels were measured with a kit from Wako Pure Chemical Industries.

**HPLC analysis for plasma lipoprotein**

Plasma lipoprotein profiles were analyzed using HPLC (Liposearch®; Skylight Biotech Inc., Tokyo, Japan) according to Okazaki et al. ⁸.

**Lipoprotein X (Lp-X) detection**

Lp-X was detected by electrophoresis as described previously ⁹. In brief, 1.5µl of fresh plasma was applied onto 1% agar gel and run in barbital buffer (pH 8.8) at 90 V for 25 min. Cholesterol was stained using a commercial reagent (Titan gel S-cholesterol, Helena laboratory).

**Measurement of hepatic lipids synthesis in vitro using liver slices**

Hepatic lipids synthesis was examined in vitro using liver slices as described elsewhere ¹⁰. In brief, the animals were killed and the liver was immediately removed and chilled. Liver slices (~100 mg) were cut into small pieces and placed in 5.0 ml of Krebs’ bicarbonate buffer (pH 7.4) containing 8 mM [2-¹⁴C] acetate (0.1 µCi/µmol). The slices were then incubated 90 min at 37°C at 120 oscillation/min. They were then saponified, and the nonsaponifiable sterols were isolated by TLC. After extraction of the non-saponifiable sterols and acidification with HCl, the ¹⁴C-labeled fatty acids were extracted. The radioactivity of isolated sterols and fatty acids was measured. The results
were expressed as nmol/h/100 mg of liver wet weight.

**Liver ceramide and diglyceride levels**

Liver ceramide and diglyceride content levels were determined using the diglyceride kinase method as described previously\(^{11}\). In brief, liver was freeze-dried and dissected free of visible connective tissue. Lipids were extracted with chloroform:methanol:PBS (1:2:0.8). Diglyceride kinase (Calbiochem) and \(\gamma\)^{32}P ATP (10 mCi/mmol cold ATP) were added to lysates preincubated with \(\beta\)-octylglucoside and 1,2-dioleoyl-\(sn\)-glycerol-3-phospho-1-glycerol. The reaction was stopped after 30 min by the addition of chloroform:methanol (2:1), and extracted lipids were spotted onto TLC plates and developed in chloroform:acetone: methanol:acetic acid:water (100:40:30:20:10). \[^{32}\text{P}\] phosphatidic acid (corresponding to diglyceride content) and ceramide-1-phosphate (corresponding to ceramide content) were identified and scraped from the TLC plate for scintillation counting.

**Measurement of hydrogen peroxide (H\(_2\)O\(_2\)) levels**

For determination of Hydrogen peroxide (H\(_2\)O\(_2\)) levels in the mouse liver, freshly harvested tissue was homogenized on ice and subsequently centrifuged at 12,000 x g for 10 min at 4°C. Total protein (10~20µg) from the supernatant was used to measure H\(_2\)O\(_2\) by OxiSelect in Vitro ROS/RNS Assay kit (Cell biolab).
**Measurement of caspase 3 and caspase 8 activity in vivo**

For determination of caspase 3 and caspase 8 activity in the mouse liver, freshly harvested tissue was homogenized on ice and subsequently centrifuged at 12,000 ×g for 10 min at 4°C. Total protein (100 µg) from the supernatant was used to measure caspase activities by the ApoAlert caspase fluorescent-assay kit (CLONTECH).

**Histological analyses**

Livers were fixed in 10% neutral buffered formalin. Paraffin-embedded sections were stained with hematoxylin and eosin (HE) staining and TdT-mediated dUTP-biotin nick-end labeling (TUNEL). Ki-67 antibody which recognizes a nuclear protein associated with cell proliferation and type 4 collagen antibody which recognizes a liver fibrous structural protein. Frozen sections of liver tissue were stained with oil-red O and examined by light microscopy.

**Mevalonate supplementation**

Male (n=5) and female (n=5) L-HMGCR KO mice were given mevalonate (Sigma) in drinking water at a concentration of 5 mM from the age of 28 days to 35 days. The mice were then killed and blood and liver samples were collected and analyzed.

Male (n=8) and female (n=7) L-HMGCR KO mice were given mevalonate from the age
of 28 days to 40 days and their survival was observed.

**Glucose supplementation**

L-HMGCR KO mice were given D-glucose (WAKO) in drinking water at a concentration of 20% (w/v) from the age of 28 days to 40 days and their survival was observed. At the age of 35 days, the survivor’s blood were collected and analyzed.

**Statistics.**

Statistical analyses were performed using Student's t test (2-tailed) as described in the Table legends. All calculations were performed with GraphPad Prism 4.0 software (GraphPad).

**Supplemental References**


7. Sato R, Goldstein JL, Brown MS. Replacement of serine-871 of hamster 3-hydroxy-3-methylglutaryl-CoA reductase prevents phosphorylation by AMP-activated kinase and blocks inhibition of sterol synthesis induced by ATP


Supplemental table I

Real-time PCR primer/probe sequences.

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Legends for supplemental figures

Supplemental figure I

Targeting strategy and conditional deletion of HMGCR gene in mice.

Cre-mediated excision of the sequences between loxP sites deleted exons 2 to 4. The location of the probe used for Southern blot and Northern blot analysis is denoted by the
horizontal filled rectangle labeled “probe for Southern blot.” or “probe for Northern blot”.

**Supplemental figure II**

(A) Total RNA from the skin, intestine and adrenal which are the cholesterogenic organs of mice (n = 5 in each group) was subjected to quantitative real-time PCR for HMGCR gene expressions as described in Supplemental Methods. Each value represents the amount of mRNA relative to that in the control mice. (B) The HMGCR mRNA expression levels from the livers of survivor L-HMGCR KO mice at 14 weeks of age. Each value represents the amount of mRNA relative to that in the control mice, which is arbitrarily defined as 1 (n=3 in each group). Values are mean ± SD.

(C) The HMGCR mRNA expression level in isolated parenchymal cells and non-parenchymal cells at 3 weeks of age (n =9 in each group). Each value represents the amount of mRNA relative to that in the parenchymal cells from control mice, which is arbitrarily defined as 1. Values are mean ± SD. Significant differences compared with control mice: **, P<0.001. (D) The HMGCR activities in isolated parenchymal cells from the control and L-HMGCRKO mice at 3 weeks of age (n =9 in each group). Values are mean ± SD. Significant differences compared with control mice: *, P<0.05.
**Supplemental figure III**

Plasma lipoprotein profiles using HPLC analysis. The chromatographic patterns of mean value from control (gray line) and L-HMGCRKO (black line) at 3 weeks of age (A and B) and 5 weeks of age (C and D) are shown. Immunoblot analysis of plasma apolipoprotein (Apo) B (E, at 3 weeks; G, at 5 weeks of age) and Low density lipoprotein receptor (LDLR) (F, at 3 weeks; H at 5 weeks of age) of control and L-HMGCRKO mice are shown. The membrane protein transferrin receptor (TfR) was used as a loading control for LDLR protein levels. (I) Representative gel electrophoresis of Lipoprotein-X (Lp-X) is shown in an agar gel of plasma samples from human serum control, fHMGCR, and L-HMGCRKO mice. Plasma of L-HMGCRKO mice contains a characteristic band for Lp-X of cathodally migrating on agar gel, indicated by the arrow. O, Origin; +, anode side of gel; −, cathode side of gel.

**Supplemental figure IV**

HE stained liver section from control (A and C) and L-HMGCRKO (B and D) mice (A and B, 4 weeks of age; C and D, 14 weeks of age).
**Supplemental figure V**

The liver tissue lysates in control and L-HMGCRKO (n = 5 in each group) were used to measure caspase 3 (A), caspase 8 (B) activity and hydrogen peroxide (H₂O₂) levels which is reactive oxygen species (C) by fluorescent assay as described in Supplemental Methods. (D) Total RNA from the livers (n = 5 in each group) were subjected to quantitative real-time PCR for apoptosis-related gene expressions at 5 weeks of age as described in Supplemental Methods. Each value represents the amount of mRNA relative to that in the control mice, which is arbitrarily defined as 1 (A, B and D respectively). Values are mean ± SD. Significant differences compared with control mice: *, P<0.05.

**Supplemental figure VI**

Male (n=15) and female (n=13) L-HMGCR KO mice were given glucose from the age of 28 days to 40 days and the survival curves were generated by the Kaplan-Meier method.
Supplementary Figure IV
Caspase 8 activity (relative fluorescence units)

Caspase 3 activity (relative fluorescence units)

Relative mRNA levels (normalized to β-actin)

Supplementary Figure V

A

B

C

D

H2O2 (µM/mg protein)

fHMGCR L-HMGCRKO

fHMGCR L-HMGCRKO

fHMGCR L-HMGCRKO

fHMGCR L-HMGCRKO

Bcl2 BAX Survivin CHOP

0 1 2 3 4 5 6 7 8 9 10

0 0.5 1.0 1.5

0 250 500 750 1000

Supplementary Figure V

A

B

C

D

H2O2 (µM/mg protein)

fHMGCR L-HMGCRKO

fHMGCR L-HMGCRKO

fHMGCR L-HMGCRKO

fHMGCR L-HMGCRKO

Bcl2 BAX Survivin CHOP

0 1 2 3 4 5 6 7 8 9 10

0 0.5 1.0 1.5

0 250 500 750 1000