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 HMHCR. 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 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.

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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].

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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[3]. 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 detention 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.*[4]. 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

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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[5]. 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 ^{32}P-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

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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[6]. 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 HMGCR[7] was kindly provided by Dr.YK Ho, MS Brown and JL Goldstein. Addional 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

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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 dithiothreitol, 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}C] mevalonate was isolated by TLC and measured using [^{3}H] mevalonate as an internal standard. HMGCR activity is expressed as picomoles of [^{14}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

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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 eletrophoresis 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-^{14}C] acetate (0.1 μCi/μmol). The slices were then incubated 90 min at 37℃ 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 ^{14}C-labeled fatty acids were extracted. The radioactivity of isolated sterols and fatty acids was measured. The results

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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 (Calbiovhem) and γ-[^{32}P] ATP (10 mCi/mmol cold ATP) were added to lysates preincubated with β-octylglucoside and 1,2-dioleoyl-*sn*-glycero-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}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_2O_2) levels

For determination of Hydrogen peroxide (H_2O_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_2O_2 by OxiSelect in Vitro ROS/RNS Assay kit (Cell biolab).

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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

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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).

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Supplemental table I

Real-time PCR primer/probe sequences.

gene	Forward primer	Reverse primer	Probe
SREBP1a	GGCCGAGATGTGCGAACT	TTGTTGATGAGCTGGAGCATGT	AGCGGTTTTGAACGACAT
SREBP1c	TGGATTGCACATTTGAAGACATG	GGCCCGGGAAGTCACTGT	CAGCTCATCAACAACCA
SREBP2	CCGGTCCTCCATCAACGA	TGGCATCTGTCCCCATGACT	AAAATCATAGAGTTGAAGGACT
LDL receptor	AGGCTGTGGGCTCCATAGG	TGCGGTCCAGGGTCATCT	TATCTGCTCTTCACCAACC
HMGCR	CGTCATTCATTTCCTCGACAAA	AGCAGAAAAAAGGGCAAAGCT	AACTGACAGGCTTAAAT
SS	CCAACTCAATGGGTCTGTTCCT	TGGCTTAGCAAAGTCTTCCAACT	CAGAAAACAAATATCATTCG
CYP7A1	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA	CATCAAGGAGGCTCTG
ACC	GGACAGACTGATCGCAGAGAAAG	TGGAGAGCCCCACACACA	TGGCAGGAGATCGCAGT
FAS	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT	ACTCGGCTACTGACACGA
SCD1	TCCGGAAATGAACGAGAGAAGGTGAAGA	AGATCTCCAGTTCTTACACGACCAC	GACGGATGTCTTCTTCCAGGTG
SCD2	GTGTCCAGGGCTGCTGTCTT	CACTCAGCCGCTCTTGCA	CCAGGATTAAGAGAACTGGA
DGAT1	TCCGCCTCTGGGCATTC	GAATCGGCCCACAATCCA	CCATGATGGCTCAGGTCCCACTGG
DGAT2	TGGAACACGCCCAAGAAAG	CACACGGCCCAGTTTCG	TGGCAGGAGATCGCAGT
MTP	GCTCCCTCAGCTGGTGGAT	CAGGATGGCTTCTAGCGAGTCT	ACCTCTGCTCAGACTC
LXRα	GCTCTGCTCATTGCCATCAG	TGTTGCAGCCTCTCTACTTGGA	CTGCAGACCGGCCCA
BAX	GGCCTTTTTGCTACAGGGTTT	GTGTCTCCCCAGCCATCCT	ATCCAGGATCGAGCAGG
Bcl2	ATCTTCTCCTTCCAGCCTGAGA	ACGTCCTGGCAGCCATGT	CAACCCAATGCCCG
CHOP	CATCCCCAGGAAACGAAGAG	GCTAGGGACGCAGGGTCAA	AAGAATCAAAAACCTTCACTACT
β actin	CGATGCCCTGAGGCTCTTT	TGGATGCCACAGGATTCCA	CCAGCCTTCCTTCTT

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

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horizontal filled rectangle labeled “probe for Southern blot.” or “probe for Nouthern 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 surviver 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: *, $P<0.05$.

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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).

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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 (H_2O_2) 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.

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Supplementary Figure I

Probe for Nouthern blot

Probe for Southern blot

ATG

6.3kb

K B E E E B E E X B B E X B

Wild-type Allele

1 2 3 4 5 6 7 8 9 10

Targeting Construct

K B E E E B E E X B B B E E E

HSV-TK HSV-TK

Neo

1 2 3 4 5

7.7kb

K B E E E B E E X B B B E E E X B

Floxed Allele

Neo

1 2 3 4 5 6 7 8 9 10

+ Cre recombinase

K B E E E B E E X E E X B

Disrupted Allele

1 5 6 7 8 9 10

12kb

: Exon

: loxP

K: *Kpn*I, B:*Bam*HI, E:*Eco*RI, X: *Xho*I

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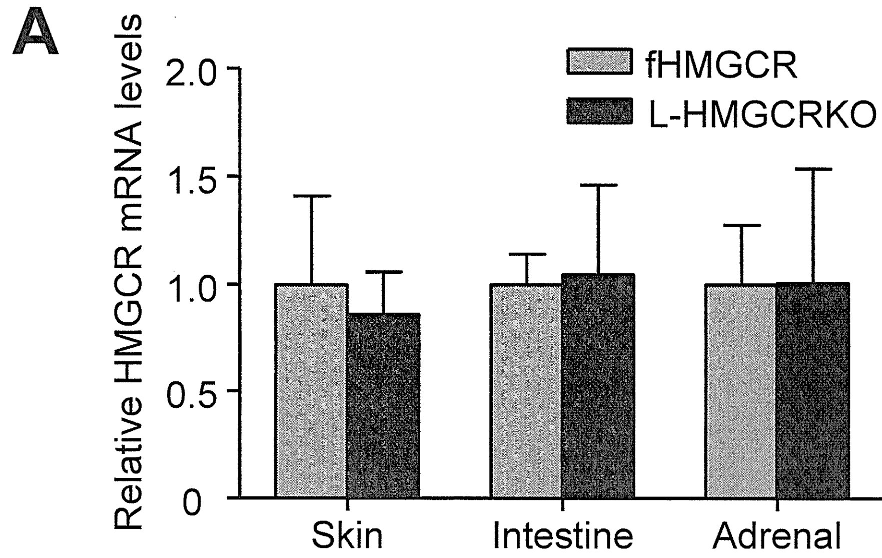
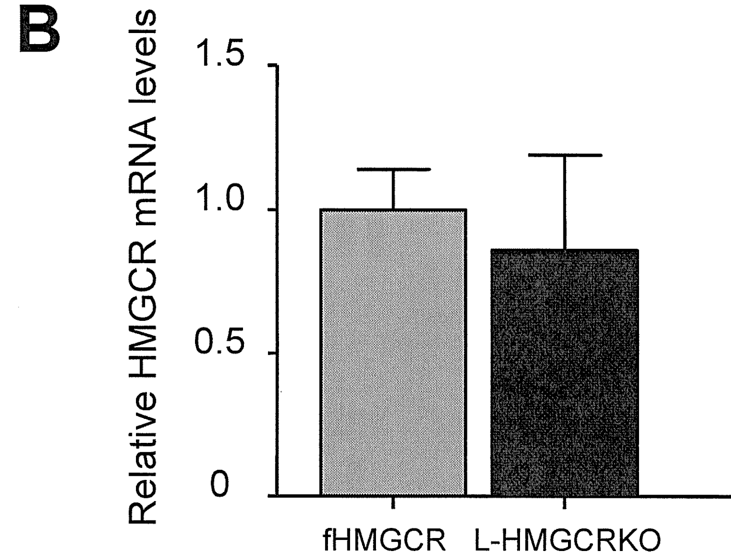
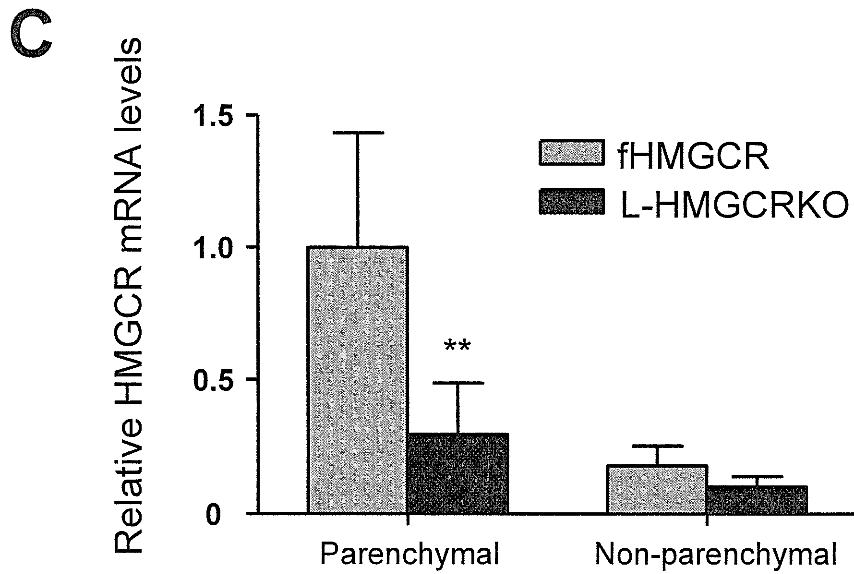
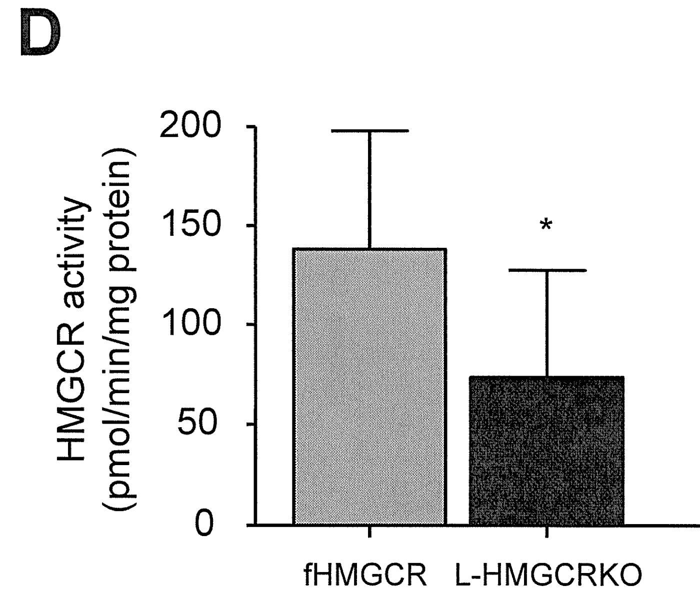
Supplementary Figure II









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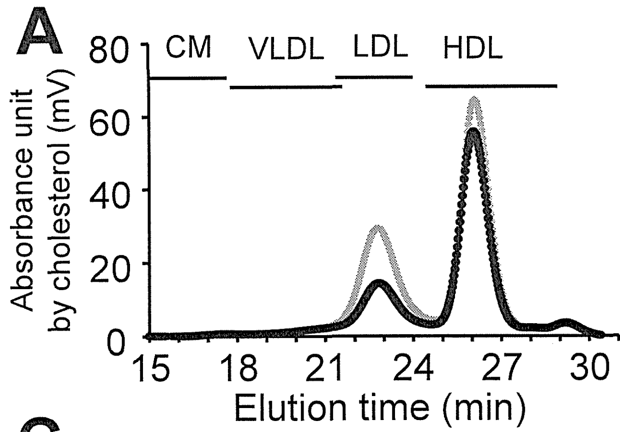
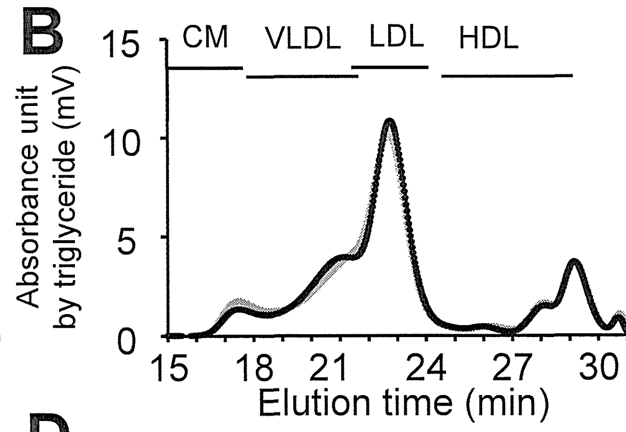
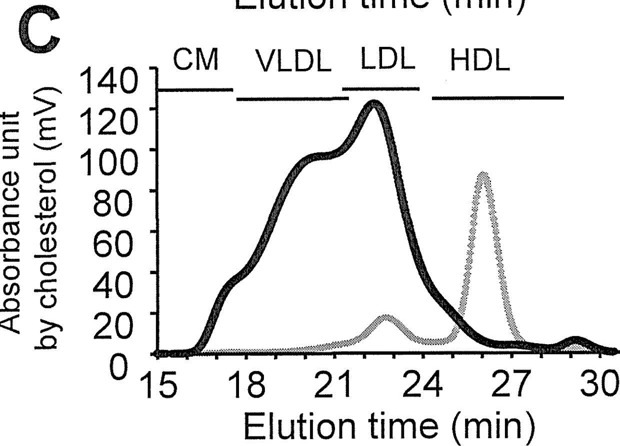
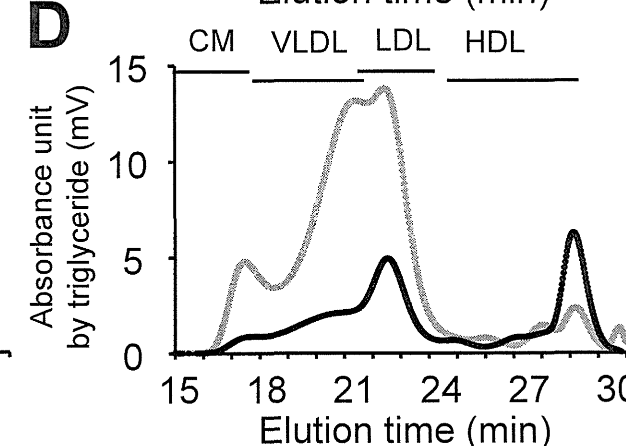
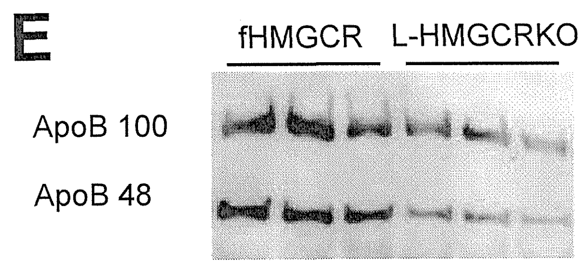
Supplementary Figure III

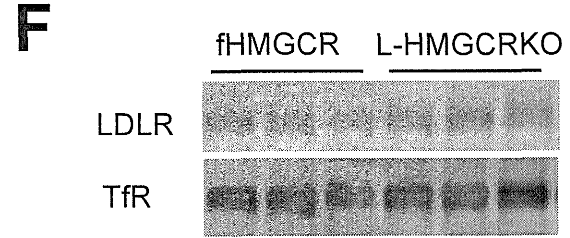


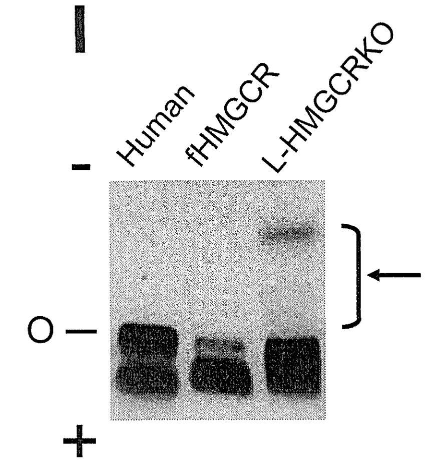


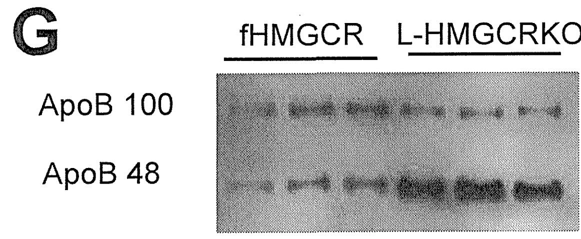


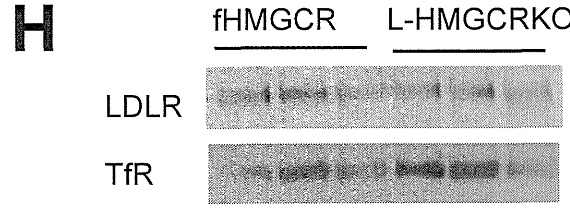












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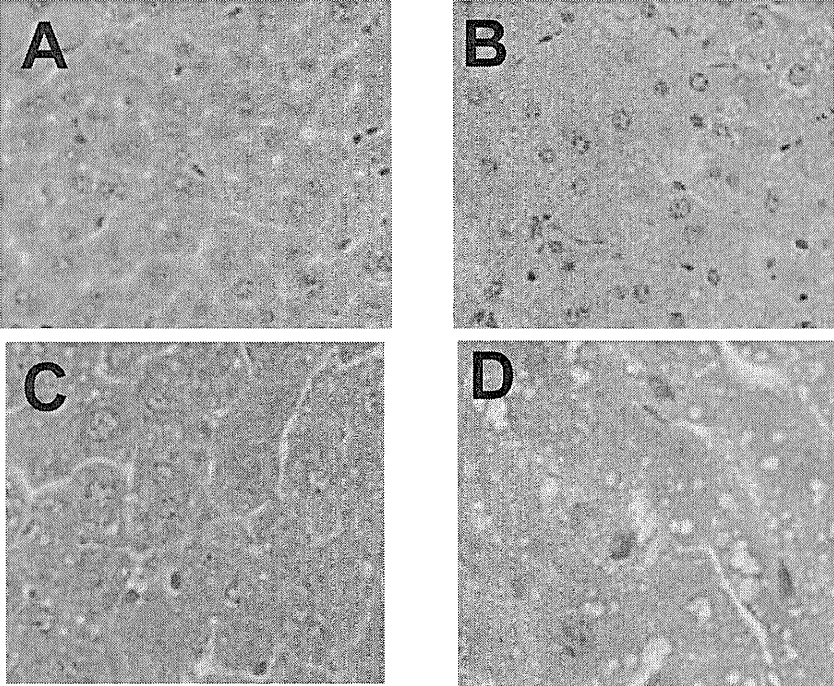
Supplementary Figure IV



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