Supplemental materials and methods

Generation of mice heterozygous for the floxed SS allele

A conditional targeting vector of a replacement type was produced by inserting one loxP site into a BamHI site in intron 5' to exon 5 and a neomycin selection cassette along with the second loxP site into an EcoRV site in 3' to exon 5 as previously described(1) (Supplemental figure 1). The transcriptional orientation of the *neo* gene was opposite to that of the SS gene. Excision of sequences between the loxP sites by Cre recombinase deletes exons 5, which includes the catalytic site of SS. We independently electroporated JH1 ES cells (A gift from Dr. J. Herz) with the targeting vector. Recombinant clones exhibiting a single allele containing a floxed SS gene were identified by PCR using primers P1 (5'-ATACAGGGGAGTGTGCCTTTCTTGTG-3') and P2 (5'-GATTGGGAAGACAATAGCAGGCATGC -3'). All targeted clones were confirmed by Southern blot analysis using a cDNA probe containing intron 3. The targeted clones were injected into the C57BL/6J blastocysts, yielding 2 lines of chimeric mice which transmitted the floxed allele through the germ line.

Generation of liver-specific SS knock-out mice

Mice expressing Cre recombinase under the control of the albumin gene promoter (Alb-Cre) (2) were backcrossed with C57BL/6J mice more than 6 times before interbreeding. $SS^{+/f}$ (f denotes flanked by loxP) carrying one copy of the Alb-Cre transgene were interbred with $SS^{+/f}$ littermates lacking Cre to generate liver-specific SS knock-out ($SS^{f/f}$ Alb-Cre; L-SSKO) mice and littermate control [$SS^{f/f}$ (fSS), $SS^{+/+}$ Alb-Cre, and $SS^{+/+}$ (WT)] mice. Age-and sex-matched littermates were used as the controls. Disruption of the floxed SS 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 SS genotyping, we used as primer A, 5' TTGACTGTCAGCAGCATGTC 3'; and primer B, 5' TGCCATCACCTTCATTGCAC 3'. All mice were group-housed in cages with a 12-hour light/dark cycle and fed CE-2 (Japan CLEA) which contained 1.0% crude carbohydrate, 24.9% crude protein and 4.6% crude fat. Unless otherwise stated, they fed a chow diet *ad libitum*, and tissues were collected in the early dark phase at a time when cholesterol biosynthesis activity is at its peak of diurnal rhythm(3). All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at Jichi Medical University and University of Tokyo.

Northern blot analysis and Quantitative real-time PCR

Total RNA was prepared from mouse tissues using TRIzol (Invitrogen). For Northern blot analysis, pooled total RNA was subjected to 1% agarose gel electrophoresis in the presence of formalin and transferred to Hybond N^+ membranes (GE healthcare). The membranes were hybridized to ^{32}P-labeled SS cDNA probes. Radioactivity was quantified with BAS 2000 (Fujifilm).

For Quantitative real-time PCR, all reactions were done in triplicate and the relative amounts of mRNAs were calculated using the standard curve method or comparative CT method with the 7300 Real-Time PCR system (Applied Biosystems) according to the manufacturer's protocol. Mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or ß actin mRNA was used as the invariant control.

The primer-probe sets for real-time PCR was shown in Supplemental table 1.

Immunoblot analyses

To prepare membrane and cytosol fractions for immunoblot analyses, aliquots of frozen liver (~100 mg) were homogenized in 1 ml buffer (20 mM TrisHCl at pH 7.4, 2 mM $MgCl_2$, 0.25M sucrose, 10 mM sodium EDTA and 10 mM sodium EGTA) supplemented with protease

inhibitor cocktail (Sigma). The liver homogenate was centrifuged at 1,000 × *g* for 5 minutes at 4°C. The supernatant was removed and used to prepare a membrane and cytosol fraction as previously described(4).

After aliquots of the membrane fraction were removed for measuring protein concentration with the BCA Kit (Pierce Biotechnology), the remainder of each sample was mixed with 4× SDS loading buffer (12% (w/v) SDS, 0.02% bromophenol blue, 30% glycerol, 0.15 M Tris-HCl at pH 6.8, and 6% ß-mercaptoethanol). Protein from the livers of mice (45 µg) was subjected to SDS-PAGE and immunoblot analysis. For immunoblot analysis of apolipoprotein (apo) B, 2 µl of plasma were delipidated and subjected to SDS-PAGE. Rabbit polyclonal antibody for mouse HMGCR was provided by Dr. YK Ho, MS Brown and JL Goldstein(5). Additional antibodies are as follows: SS (S10120, BD bioscience), protein disulfide isomerase (PDI) (SPA-891, Stressgen), apoB (SC-11795, Santa Cruz Biotechnology), LDLR (AF2255, R and D Systems) and SR-BI (NB400-104SS, Novus biologicals)

Assays of SS and HMGCR activity

Liver were homogenized in a buffer containing 15 mM nicotinamide, 2 mM $MgCl_2$, and 100 mM potassium phosphate, pH 7.4, and centrifuged at 10,000 × *g* for 20 minutes at 4 °C. The supernatants were centrifuged at 105,000 × *g* for 1 hour at 4 °C, and the resultant pellets, comprising a microsomal fraction, were washed, resuspended in the same buffer, and stored in aliquots at -80 °C. SS and HMGCR activities were measured as described previously(6, 7) with slight modifications with respect to use of thin-layer chromatography (TLC) to separate [^{3}H] squalene(8).

Lipids and biochemical analysis

Blood was drawn from the retro-orbital sinus. Plasma was separated immediately and stored at –80°C. Plasma lipids, lipoproteins and ALT were measured as previously described(7). Hepatic squalene content was determined by the HPLC method at the TORAY Research Center **(**Tokyo**,** Japan**)**. Hepatic mevalonate content was determined by the HMGCR enzymatic cycling assay at the Asahi Kasei Pharma Corporation (Shizuoka, Japan)(9). Hepatic FPP was determined by LC-MS/MS as previously described(10). Hepatic sterol intermediates and oxysterols were determined by LC-MS/MS as previously described(11)

Measurement of hepatic lipids synthesis in vitro using liver slices

Hepatic lipids synthesis *in vitro* using liver slices was measured as previously described(7). Non-saponifiable farnesol from livers were separated by TLC with diethyl ether/ hexane/ chloroform/ acetic acid **(**550:400:50:1, vol/vol) (8)**.**

Measurement of hepatic cholesterol synthesis in vivo

Mice were fed *ad libitum* a regular chow diet prior to the experiment. At the early dark cycle, the mice were anesthetized and intravenously injected with 0.9% saline containing ^{3}H water via jugular vein. The amount of ^{3}H water was 4 mCi/100g body weight(12). One hour later the livers were removed for measurement of ^{3}H-labeled digitonin-precipitable sterols as previously described(13)

Measurement of intestinal cholesterol absorption

Intestinal cholesterol absorption was measured by a fecal isotope ratio method using [4-^{14}C] cholesterol and [5,6-^{3}H] sitostanol as previously described(14). Non-fasted mice were dosed with 0.5μCi of ^{14}C-labeled cholesterol and 1μCi of ^{3}H-labeled sitostanol in corn oil. Feces were collected for 48 hours after dosing, and the ratio of ^{14}C to ^{3}H in aliquots of samples was used to calculate the percent cholesterol absorption.

Measurement of in vivo VLDL secretion rate

In vivo VLDL secretion rates were estimated by using Triton WR1339 to inhibit lipoprotein lipolysis as described previously(15). Plasma levels of triglyceride were measured at 1, 2, 3, and 4 hours after intravenously injection of Triton WR 1339.

Measurement of ^{125}I-labeled Rabbit β-VLDL kinetics

Plasma lipoprotein kinetics were measured as previously described(16). In brief, mice were anesthetized with sodium pentobarbital and received an intravenous injection of 0.25 ml of 0.15 M NaCl containing bovine serum albumin (2 mg/mI) and 10 μg of ^{125}I-labeled rabbit ß-VLDL (293 cpm/ng protein). Blood was collected at the indicated time by retro-orbital puncture. The plasma content of ^{125}I-labeled apoB was measured by isopropanol precipitation followed by gamma counting.

Histology

Liver slices were stained with Hematoxylin and eosin (H&E), oil red O and TdT-mediated dUTP-nick end labeling (TUNEL), Immunostaining was performed for Ki-67(7).

Other assays

Caspase-3 activity were determined as described previously(7).

Statistics

Statistical analyses were performed using the Student's *t* test (2-tailed) or two-way repeated-measures ANOVA as described in Table and figure legends.

Supplemental table and figure legends

Supplemental table 1. Primer and probe sequences for quantitative real-time PCR.

Supplemental table 2. Plasma lipids and ALT levels in female mice.

Blood samples were taken from male mice fed a normal chowdiet adlibitum or fasted for 16 hours before the study. Each value represents the mean ± SD. *$p < 0.05$ vs. fSS mice with the same age by Student's *t* test.

Supplemental figure 1. **Targeting strategy to disrupt the SS gene in mice.**

The sequences between *loxP* sites exon 5 are designed to be deleted by Cre-recombinase. The location of the probe used for Southern blot and Northern blot analysis is denoted by the horizontally filled.

Supplemental figure 2. **Deletion of SS gene in the livers and intestinal cholesterol absorption.**

(A) Southern blot analysis. Ggenomic DNA was prepared from the livers of WT, fSS and L-SSKO male mice at the ages of 4 weeks. **(B)** Northern blot. Total RNA was prepared from the livers of

control and L-SSKO male at the ages of 12 weeks. **(C)** Cholesterol absorption was determined in the male mice at the age of 12 weeks (fSS, n = 4; L-SSKO, n = 5). Each value represents the mean ± SD.

Supplemental figure 3. **Synthesis and hepatocellular toxicity of farnesol.**

A) Time course of synthetic rate of farnesol from ^{14}C-acetate in the whole liver slices (n = 5 in each group). Each value represents the mean ± SD. *$p < 0.05$ by Student's *t* test. **(B**) Hepatocellular toxicity of increasing concentrations of farnesol. Primary hepatocytes were prepared from C57BL/6J mice (n = 3), seeded onto collagen-coated 96-well plate at a density of 1.8×10^4 cells per well and cultured. The cells were treated with various concentrations of trans,trans-farnesol (Sigma) for 7 days. Values are mean ± SD of triplicates for each treatment. Cytotoxicity was determined by MTT assay kit (Promega) according to the manufacturer's instructions. **$p < 0.001$ vs 0 μM of farnesol by Student's *t* test. **(C)** Effects of feeding with high fat high sucrose diet (HFHSD) on plasma ALT levels. At 12 weeks of age, mice were fed HFHSD (60% kcal in fat, 20% kcal in carbohydrate and 20% kcal in protein; #D12492, Research Diets) which is known to induce hepatic steatosis and dysfunction. Plasma ALT levels were measured before and 12 weeks after the feeding in fSS and L-SSKO male mice (fSS, n = 9; L-SSKO, n = 5). Each value represents the mean ± SD. *$p < 0.05$ by Student's *t* test.

Supplemental figure 4. **Hepatic contents of reguratory oxysterols.**

The tissue lysates from the livers of male mice at the age of 12 weeks (n = 5 in each group) were used for measurements of oxysterols. Each value represents the mean ± SD. *$p < 0.05$ Student's *t* test.

Supplemental figure 5. Schematic view of hepatic cholesterol metabolism in L-SSKO mice.

In parentheses, upward-facing arrows, increased; downward-facing arrows decreased; right-facing arrows, unchanged. ApoB indicates apolipoprotein B; CideB, cell death-inducing DNA fragmentation factor 45-like effector B; FPP, farnesyl diphosphate; FPPS, FPP synthase; GGPP, GGPP,geranyl geranyl diphosphate; HMGCR, HMG-CoA reductase; LDLR, LDL receptor; LXR, liver x receptor. MTTP, microsomal triglyceride transfer protein; SQLE, squalene epoxydase; SREBP-1c, sterol regulatory element-binding protein 1c; SS, squalene synthase.

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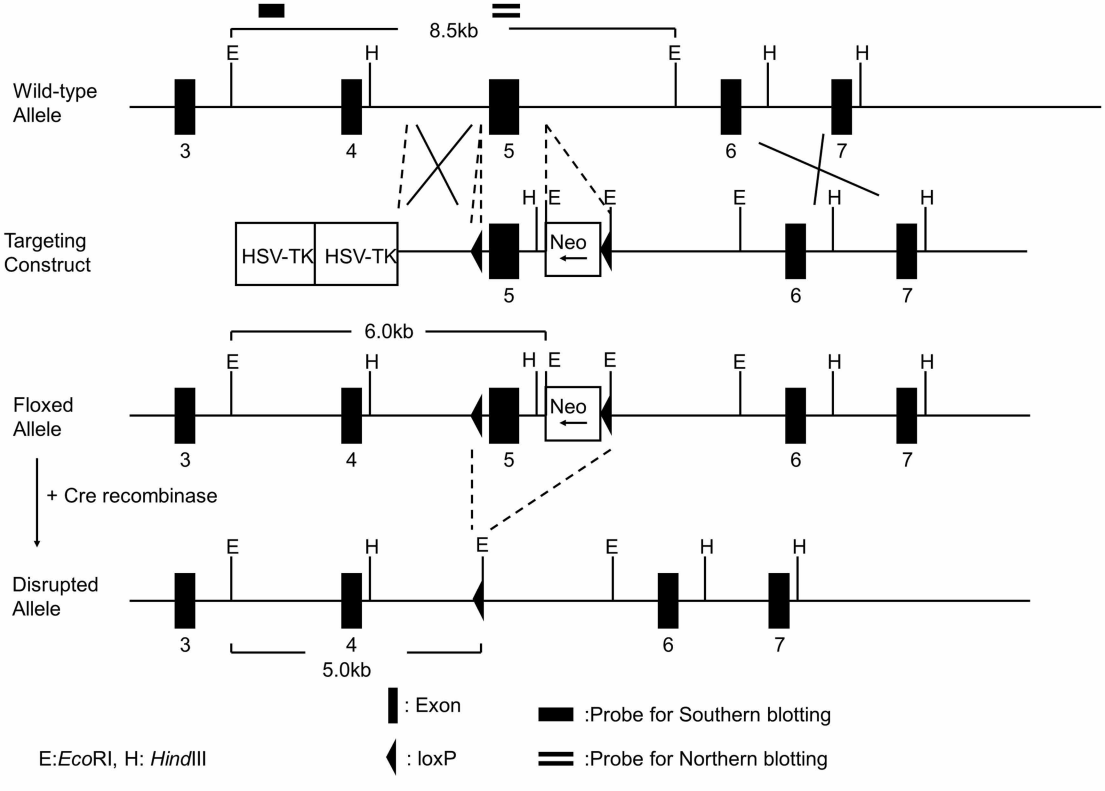
Supplemental table 1

gene	Forward primer	Reverse primer	Probe
SS	CCAACTCAATGGGTCTGTTCCT	TGGCTTAGCAAAGTCTTCCAACT	CAGAAAACAAATATCATTCG
SREBP2	CCGGTCCTCCATCAACGA	TGGCATCTGTCCCCATGACT	AAAATCATAGAGTTGAAGGACT
SREBP-1a	GGCCGAGATGTGCGAACT	TTGTTGATGAGCTGGAGCATGT	AGCGGTTTTGAACGACAT
SREBP-1c	TGGATTGCACATTTGAAGACATG	GGCCCGGGAAGTCACTGT	CAGCTCATCAACAACCA
LDLR	AGGCTGTGGGCTCCATAGG	TGCGGTCCAGGGTCATCT	TATCTGCTCTTCACCAACC
PCSK9	TTGCAGCAGCTGGGAACTT	CCGACTGTGATGACCTCTGGA	ACGACGCCTGCCTCTACTCCCCAG
LRP1	AACTTGCAGCCCTAAGCAGTT	GCAATCTCTTTCACCGTCACA	CCAAATCACCTGTATCTCA
SR-BI	TCCCTCATCAAGCAGCAGGT	TTCCACATCCCGAAGGACA	TCAAGAATGTCCGCATAGA
HMGCR	CGTCATTCATTTCCTCGACAAA	AGCAGAAAAAAGGGCAAAGCT	AACTGACAGGCTTAAAT
FPPS	ATGGAGATGGGCGAGTTCTTC	CCGACCTTTCCCGTCACA	CTACCTTGATCTCTTTGGAG
SQLE	GGCTCAGGCCTTGTATGAATTATT	GCTTTTCGGAGCTGATGCA	TCAGCTACAGATGATTCC
FAS	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT	ACTCGGCTACTGACACGA
MTTP	GCTCCCTCAGCTGGTGGAT	CAGGATGGCTTCTAGCGAGTCT	ACCTCTGCTCAGACTC
ApoB	CGTGGGCTCCAGCATTCTA	TCACCAGTCATTTCTGCCTTTG	CCAATGGTCGGGCAC
CideB	Taqman® Gene Expression Assays (Assay ID: Mm00438213_m1) purchased from Applied Biosystems.		
ABCA1	TGCCACTTTCCGAATAAAGC	GGAGTTGGATAACGGAAGCA	ATGCCGTCTGCAGGAA
LXR-α	GCTCTGCTCATTGCCATCAG	TGTTGCAGCCTCTCTACTTGGA	CTGCAGACCGGCCCA
CYP7A1	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA	CATCAAGGAGGCTCTG
ABCG5	TGGCCCTGCTCAGCATCT	ATTTTTAAAGGAATGGGCATCTCTT	CTGCTTATTGGATCTGGATT
ABCG8	CCGTCGTCAGATTTCCAATGA	GGCTTCCGACCCATGAATG	TTCCGGGACCTGCCCA
ß actin	CGATGCCCTGAGGCTCTTT	TGGATGCCACAGGATTCCA	CCAGCCTTCCTTCTT
GAPDH	Taqman® Gene Expression Assays (Assay ID: Mm99999915_g1) purchased from Applied Biosystems.		

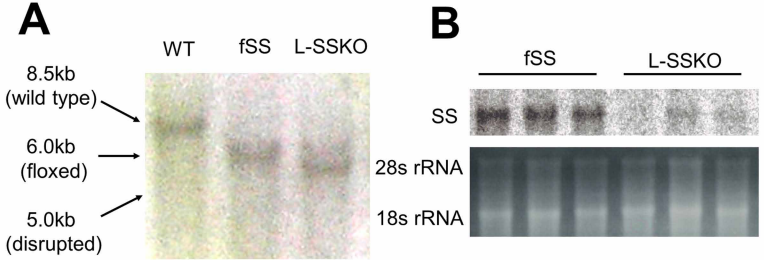
Supplemental table 2

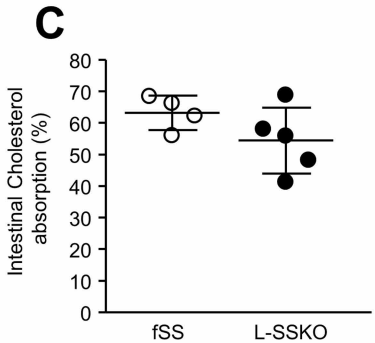
Age (weeks)	12	
Genotype	fSS	L-SSKO
Plasma total cholesterol (mg/dl)		
fed	69.5±7.4 (11)	55.0±14.9 (11) *
16 hour-fasted	83.7±14.9 (6)	78.1±6.4 (5)
Plasma triglyceride (mg/dl)		
fed	62.9±34.4 (11)	35.9±16.9 (7) *
16 hour-fasted	70.2±26.0 (6)	50.8±7.8 (5) *
Plasma free fatty acid (mmol/l)		
fed	0.44±0.10 (11)	0.41±0.12 (11)
16 hour-fasted	1.93±0.17 (6)	1.76±0.14 (5)
Plasma ALT (IU/l)	10.3±3.2 (11)	46.7±70.2 (11) *

Supplemental figure 1

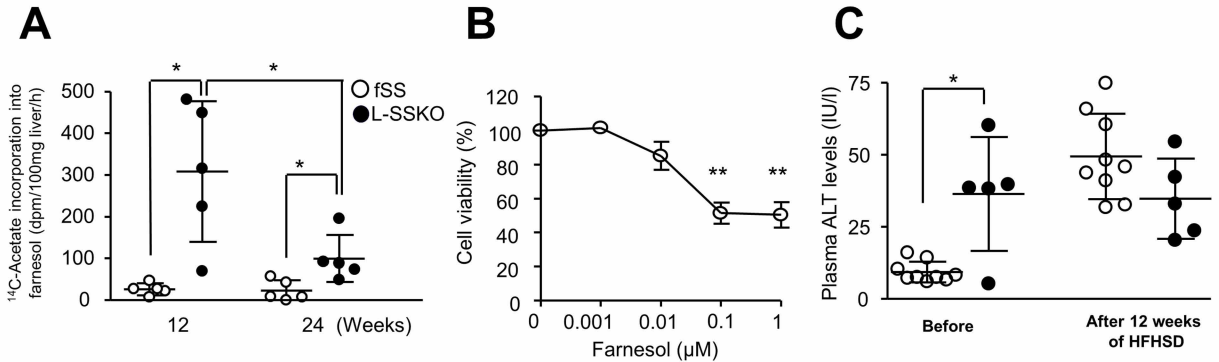


Supplemental figure 2

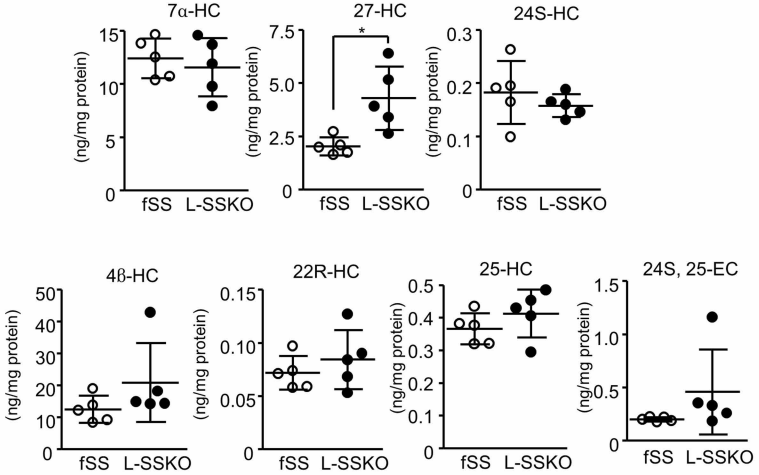




Supplemental figure 3



Supplemental figure 4



Supplemental figure 5