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Early embryonic lethality caused by targeted disruption of the HMG-CoA reductase gene

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Running Title: HMG-CoA Reductase Deficiency in Mice

SUMMARY

The endoplasmic reticulum (ER) enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which converts HMG-CoA to mevalonate, catalyzes the rate-limiting step in cholesterol biosynthesis. Since this mevalonate pathway also produces several non-sterol isoprenoid compounds, the level of HMG-CoA reductase activity may coordinate many cellular processes and functions. We used gene targeting to knock out the mouse HMG-CoA reductase gene. The heterozygous mutant mice (*Hmgcr*+/-) appeared normal in their development and gross anatomy and were fertile. Although HMG-CoA reductase activities were reduced in *Hmgcr*+/- embryonic fibroblasts, the enzyme activities and cholesterol biosynthesis remained unaffected in the liver from *Hmgcr*+/- mice, suggesting that the haploid amount of *Hmgcr* gene is not rate-limiting in the hepatic cholesterol homeostasis. Consistently, plasma lipoprotein profiles were similar between *Hmgcr*+/– and *Hmgcr*+/+ mice. In contrast, the embryos homozygous for the *Hmgcr* mutant allele were recovered at the blastocyst stage, but not at E8.5, indicating that HMG-CoA reductase is crucial for early development of the mouse embryos. The lethal phenotype was not completely rescued by supplementing the dams with mevalonate. Although it has been postulated that a second, peroxisome-specific HMG-CoA reductase could substitute for the ER reductase *in vitro*, we speculate that the putative peroxisomal reductase gene, if existed, does not fully compensate for the lack of the ER enzyme at least in embryogenesis.

INTRODUCTION

The mevalonate pathway produces isoprenoids that are essential for diverse cellular functions, ranging from cholesterol synthesis to growth control. The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA)[1] reductase (EC 1.1.1.34), which catalyzes the conversion of HMG-CoA to mevalonate, is the rate-limiting enzyme in the mevalonate pathway (1). Because of its major role in cholesterol biosynthesis, the regulation of HMG-CoA reductase has been intensely studied. To ensure a steady mevalonate supply, the non-sterol and sterol end-products of mevalonate metabolism exert feedback regulation on the activity of this enzyme through multivalent mechanisms, including inhibition of transcription of the HMG-CoA reductase mRNA, blocking of translation, and acceleration of protein degradation, thus regulating the amount of reductase protein over a several hundred-fold range (reviewed in Ref.1-3).

Inhibitors of HMG-CoA reductase, statins, are potent hypocholesterolemic agents which exhibit some cholesterol-independent, or so-called pleiotropic, effects, which involve improving or restoring endothelial function, enhancing the stability of atherosclerotic plaque, decreasing oxidative stress and vascular inflammation (4). Benefits of statins also extend beyond cardiovascular diseases, including a reduction in the risk of dementia (5), Alzheimer's disease (6), ischemic stroke (7), and osteoporosis (8,9). Most of these pleiotropic effects of statins are shown to be mediated by their ability to block the synthesis of non-sterol isoprenoid intermediates. However, it remains to be unraveled how the mevalonate pathway is affected in those disease processes, particularly in cells involved in atherogenesis. In addition, some of the statins' collateral effects have been found to be independent of HMG-CoA reductase (10-12).

In mammals, only one gene has been found to encode HMG-CoA reductase

(13). In yeast, fungi, and plants, on the other hand, more than one gene encode the enzyme. Yeast, for example, contains two functional genes for HMG-CoA reductase, HMG-CoA reductase 1 (*HMG1*) and *HMG2*. *HMG1* and *HMG2* are differently expressed and when *HMG1* is deleted, *HMG2* can replace the function of *HMG1* (14). Given the major role of HMG-CoA reductase in the mevalonate pathway, it is tempting to hypothesize that mammalian cells also have a second gene for the enzyme. In fact, although the classical form of the enzyme is a transmembrane-protein anchored to the endoplasmic reticulum (ER), recent studies using a mutant cell line which lacks the ER isoform of the enzyme indicate the existence of a second isoform of the reductase exclusively localized in peroxisomes and that the peroxisomal activity might be due to a second gene (15-17).

We have previously shown that the targeted disruption of the gene for squalene synthase, the first committed enzyme of sterol synthesis, results in embryonic death at midgestation with growth retardation and defective neural tube closure (18). In these mice, the non-sterol pathways are presumed to be retained, while *de novo* cholesterol synthesis is blocked. To further determine the physiological consequences of perturbation of the mevalonate pathway and gain some insights into a putative second gene for HMG-CoA reductase, we have generated and characterized mice defective in mevalonate synthesis by disrupting the gene for HMG-CoA reductase.

EXPERIMENTAL PROCEDURES

Generation of HMG-CoA Reductase Knockout Mice. A replacement-type targeting vector was constructed; the 0.8-kb short arm spanning exon 14 to 15, and the 10-kb long arm fragment encompassing exon 2 through 12 were generated by polymerase chain reaction (PCR) using genomic DNA from the 129/Sv mouse as template. Primers used were as follows: 5'-CCGCTCGAGAAAGGAGGCCTTTGATAGCACCAGCA-3' (exon 14) and 5'-CCGCTCGAGCTTAGAGATCATGTTCATGCCCATGG-3' (exon 15) for the short arm and 5'-GCGGCCGCTTTGTGGCCTCCCATCCCTGGGAAGTTATTGT-3' (exon 2) and 5'-GCGGCCGCCTCTGCATCGCTAAGGAACTTTGCACCTTTCT-3'(exon 12) for the long arm. Integrity of the amplified fragments was verified by Southern blot analysis and partial sequencing. After subcloning into pCR2.1 vector (Invitrogen, Carlsbad, CA), the fragments were cut out and inserted into the *Xho*I and *Not*I sites, respectively, of the pPolII-short-neo-bpA-HSVTK as described previously (19). Thus, a 1.2-kb region spanning the exon 12 to 14 of the HMG-CoA reductase gene was replaced by a neomycin-resistant cassette, which was expected to abolish translation of the entire carboxyl half of the protein containing the catalytic activity (20). After linearization by digestion with *Sal*I, the vector was electroporated into JH1 embryonic stem cells (A gift from Dr. J. Herz at University of Texas Southwestern Medical Center at Dallas). Targeted clones, which had been selected in the presence of G418 and 1-(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)-5-iodouracil, were identified by PCR using the following primers: primer 1, 5'-GATTGGGAAGACAATAGCAGGCATGC-3' and primer 2, 5'-GCTGGGTACGGTAGCACAGTTATGGT-3' (Fig. 1). Homologous recombination was verified by Southern blot analysis after *Eco*RI digestion using a 3'-flanking probe (Fig. 1). Targeted embryonic stem cell clones were injected into the

C57BL/6J blastocysts yielding three lines of chimeric mice, which transmitted the disrupted allele through the germ line.

All experiments reported here were performed with 129/Sv-C57BL/6J hybrid descendants (F_1 and subsequent generations) of these animals consuming a normal chow diet (MF, Oriental Yeast, Tokyo, Japan). To avoid differences resulting from diurnal changes in the reductase activity (21,22), mice were maintained on a strict 12-h light-dark cycle. One week prior to the measurement of reductase activities and sterol synthesis rates, mice were caged individually to minimize nongenetic variability (21).

Analysis of Embryos. Embryos were harvested from timed matings of heterozygous intercrosses. Genotyping of embryos was performed by Southern blotting of DNA isolated from fetal membranes or whole embryos. For genotyping of blastocysts, female heterozygote animals were superovulated with pregnant mare serum and human chorionic gonadotropin and mated to heterozygote males. Blastocysts were flushed from uteri at 3.5 days post coitum (E3.5), then lysed individually as described (23) and subjected to PCR analysis. Sense primers for mutant and wild-type allele were the primer 1 and primer 3, 5'-AGTCCCATAATCCTCTGCTTAGCTT-3', respectively. Each allele was amplified separately with a common antisense primer, primer 4, 5'-CTACATTACCCTAAGCAGGCAATGT-3' (Fig. 2A) for 40 cycles at 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 2 min. PCR products for each blastocyst were subjected to 2%-agarose gel electrophoresis followed by Southern blotting with an internal primer. Control PCR experiments with limiting dilutions of *Hmgcr*+/- tail genomic DNA template demonstrated that the sensitivities for detecting mutant or wild-type allele were comparable.

Mevalonate Supplementation. To test whether supplementation of mevalonate to pregnant females could rescue the homozygous embryos, D, L-mevalonic

acid lactone (Sigma, St. Louis, MO) was infused via a miniosmotic pump (ALZET model 2004 osmotic pump, ALZA, Palo Alto, CA), which was implanted subcutaneously in female heterozygous mice. About one week after surgery, the mice were subjected to timed matings with heterozygous males. The pumps are designed to release the content constantly for 4 weeks, long enough to cover the entire pregnancy period. In a pilot experiment, plasma concentration of mevalonate rose from 25-35 ng/ml in non-treated females to 60,000-70,000 ng/ml in infused animals.

Mouse Embryonic Fibroblasts. Primary mouse embryonic fibroblasts (MEFs) were prepared from 14.5-day-old embryos and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). MEFs were used within three passages. All experiments were done when the cells reached subconfluence. Each monolayer was washed three times with phosphate-buffered saline, after which fresh medium containing either 10% (v/v) FCS or 5 mg/ml of human lipoprotein-deficient serum (LPDS) with or without 100 nM simvastatin (Wako, Osaka, Japan) were added. Cells were incubated for 6 h and subjected to the following analyses. Simvastatin was activated by alkaline hydrolysis prior to use.

HMG-CoA Reductase Activity Assay. For preparation of liver microsomes, animals were sacrificed during the early dark cycle, at a time when HMG-CoA reductase activity was at its peak of diurnal rhythm (21). Livers 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 min at 4℃. The supernatants were centrifuged at 105,000 *g* for 1 h at 4℃, and the resultant pellets, a microsome fraction, were washed, resuspended in the same buffer, and stored in aliquots at -80℃. HMG-CoA reductase activities were measured essentially as described (24). Briefly, the microsome fractions (~50 μg) were incubated in 20 μl of a buffer containing 110

μM D, L-[3-^{14}C] HMG-CoA (4.5 μCi/μmol), 5 mM NADPH, 10 mM EDTA, 10 mM dithiothreitol, and 100 mM potassium phosphate, pH 7.4, at 37℃ for 30 min. Reaction was terminated by the addition of 10 μl of 2 N HCl and incubated for another 30 min at 37℃ to lactonize the mevalonate formed. The [^{14}C]mevalonate was isolated by thin-layer chromatography and counted using [^{3}H]mevalonate as an internal standard. For MEFs, cellular extracts were prepared as described (25). Extracts (~50 μg of protein) were incubated in 50 μl of a buffer containing 30 μM D, L-[3-^{14}C] HMG-CoA (20 μCi/μmol), 2.5 mM NADPH, 5 mM EDTA, 5 mM dithiothreitol, and 100 mM potassium phosphate, pH 7.4, at 37℃ for 120 min, then processed as in the assay of liver microsomes. HMG-CoA reductase activity is expressed as picomoles of [^{14}C]mevalonate formed per minute per mg of protein.

Measurement of Hepatic Cholesterol Synthesis. Cholesterol synthesis in the liver was estimated in littermate 12-week-old male mice (n = 6) during the mid light cycle as previously described (18,26). In brief, animals were given food and water *ad libitum* and injected intraperitoneally with [2-^{14}C]acetate (37 kBq/kg body weight). After 1 h, animals were euthanized and the liver was removed. Two portions of the liver (200-300 mg/each) were saponified, and the digitonin-precipitable sterols were isolated for the measurement of radioactivities. The results were expressed as ^{14}C dpm/100 mg wet weight of liver/h.

Northern Blot Analysis. Poly(A^{+}) RNA was isolated and pooled from the livers of five animals. 1.2 μg were subjected to 1%-agarose gel electrophoresis in the presence of formalin. The fractionated RNA was transferred to Hybond N (Amersham Biosciences, Piscataway, NJ). The filters were hybridized to ^{32}P-labeled cDNA probes: HMG-CoA reductase, HMG-CoA synthase, mevalonate kinase, farnesyl diphosphate synthase, squalene synthase, and LDL receptor. As a control for loading

of mRNA, the same filter was hybridized with a GAPDH probe. Radioactivity was quantified with a phosphorimager and the results were corrected for loading with GAPDH, and the fold-increase in each mRNA was calculated.

Other assays. The content of cholesterol and triglycerides in plasma and liver was measured as described (18,27). Lipoproteins were fractionated by high performance liquid chromatography (HPLC) as described (28).

Statistics. Data are represented as mean +/- S.D. The Student's *t* test was used to compare the mean values between two groups.

RESULTS

Embryonic Lethality of *Hmgcr*-/- Mice. A replacement-type vector, which allowed the deletion of exons 12-14 to abolish the translation of the entire carboxyl half of the enzyme which is crucial for the catalytic activity (20,29), was constructed and used to generate heterozygous HMG-CoA reductase knockout mice (*Hmgcr*+/-). The heterozygous mutant mice appeared normal in their development and gross anatomy, and were fertile. Intercrosses between heterozygotes yielded no viable offspring homozygous for the mutant *Hmgcr* allele (*Hmgcr*-/-) (+/+:+/-:-/- = 52:106:0, $p < 0.0001$, χ^2 test) (Table I), indicating that *Hmgcr* expression is essential for the development and survival of embryos. To determine the developmental stage where the embryos were lethal, timed pregnancies of *Hmgcr*+/- matings were examined at E8.5 and 13.5 of gestation. No *Hmgcr*-/- embryos were identified at these time points (Table I). In addition, no resorbed embryos were detected, suggesting that death of *Hmgcr* null embryos occurs very early in the development, potentially prior to implantation. We therefore analyzed preimplantation embryos. Superovulated *Hmgcr*+/- females were mated with *Hmgcr*+/- males and blastocysts were isolated at E3.5. Blastocysts were genotyped directly by using PCR (Fig. 2). Among 74 phenotipically normal E3.5 blastocysts, 19 (26%) were found to be homozygote (Table I, Fig. 2B). Therefore, at the blastocyst stage, *Hmgcr*-/- embryos were viable and identified at the expected Mendelian frequency. *Hmgcr*-/- blastocysts were morphologically indistinguishable from *Hmgcr*+/- or +/+ embryos.

Attempt to Rescue the Homozygotes by Mevalonate Supplementation. If the embryonic lethality of the homozygotes results from the deficiency of mevalonate and subsequent sterol and non-sterol products of the mevalonate pathway, it is conceivable that supplementation of mevalonate would reverse the phenotype. To test

this hypothesis, we supplemented the pregnant *Hmgcr*+/- mice with mevalonate using an osmotic pump which was implanted subcutaneously. Although this intervention yielded a marked increase in the maternal plasma mevalonate level, no viable *Hmgcr*-/- offspring was obtained among 31 mice from 5 litters (Table II).

To determine whether mevalonate supplementation allows null embryos to develop beyond implantation, we studied embryos at E9.5-10.5. Ten out of 49 (20%) decidua were remarkably smaller than the others and contained embryos with appearance corresponding to those at E5.5-6.5 (the egg cylinder stage). The remaining 39 normal-sized decidua contained phenotypically normal embryos, none of which were genotyped as homozygote (Table II). *Hmgcr*+/+ female mice, which were supplemented with mevalonate, mated to the same *Hmgcr*+/- males and examined at E9.5, gave rise to 20 decidua with equivalent size which contained properly developed concepti. These results suggest that mevalonate supplementation of the pregnant females enabled *Hmgcr*-/- embryos to implant but failed to support the development after egg cylinder stage.

Analysis of Heterozygous Mice. The *Hmgcr* mRNA levels in *Hmgcr*+/- mice were slightly reduced in the liver (Fig. 3). Expression levels of four genes in the cholesterol biosynthetic pathway, both upstream and downstream of HMG-CoA reductase (encoding HMG-CoA synthase, mevalonate kinase, farnesyl diphosphate synthase, and squalene synthase) showed a 50-70% increase in the liver of heterozyogotes, while the expression of the gene for LDL receptor remained unaffected. On the other hand, there was no significant change in the hepatic HMG-CoA reductase activities. Consistently, the amounts of [^{14}C]acetate incorporated into digitonin-precipitable sterols in the liver were not different between the wild-type and heterozygous mice (Table III).

Analysis of hepatic lipids demonstrated that both cholesterol and triglyceride content were unchanged. Plasma levels of total cholesterol and triglycerides did not differ in the two groups of mice. Plasma lipoprotein analysis by HPLC revealed no discernible difference in the amount of each lipoprotein fraction (data not shown).

***Hmgcr*+/- MEFs**. To investigate the regulation of HMG-CoA reductase in *Hmgcr*+/- mice further, we isolated MEFs from *Hmgcr*+/- and *Hmgcr*+/+ embryos. Their growth in culture media was comparable. Fig. 4 summarizes HMG-CoA reductase activities of MEFs maintained for 6 h in the presence of either 10% FCS, or 5 mg/ml LPDS plus/minus 100 nM simvastatin, a competitive inhibitor of HMG-CoA reductase, which induces upregulation of HMG-CoA reductase *in vivo* and *in vitro*. As expected, the enzyme activity in MEFs was upregulated by the addition of LPDS, and further induced by concomitant treatment with simvastatin. In each experimental condition, the HMG-CoA reductase activity was reduced by approximately 50% in *Hmgcr*+/- MEFs. Increases in the enzymatic activities over the levels observed in the presence of FCS were approximately two- and twelve-fold in the presence of LPDS and in the presence of LPDS plus simvastatin, respectively, and did not differ significantly between heterozygous and wild-type MEFs.

DISCUSSION

In the present study, we have demonstrated that HMG-CoA reductase is essential for the early development of the embryos. Null embryos were recovered at the blastocyst stage, but not at E8.5, indicating that loss of HMG-CoA reductase activity through targeted disruption of the gene in the germ line leads either to implantation failure or to embryonic death prior to implantation. Moreover, the lethal phenotypes of *Hmgcr*-/- embryos were not completely reversed by supplementation with mevalonate to the dams.

Mammalian cells accelerate their growth rate dramatically upon implantation. Cholesterol synthesis in the embryos appears to begin at the peri-implantation stage (around E4-5 in mice) (30,31). Cholesterol plays an essential role in mammalian embryonic development, including the covalent modification of the morphogenic sonic hedgehog signal pathway during early gestation (32). Nevertheless, defective cholesterol synthesis is unlikely to explain the peri-implantational lethality of *Hmgcr*–/– embryos, because mice lacking squalene synthase (*Ss*-/-), the first committed enzyme of sterol synthesis in the mevalonate pathway, are viable until around E9.5 even with gross growth retardation and defective neural tube closure (18). Observations in *Ss*-/- embryos suggest that even in the complete absence of endogenous cholesterol biosynthesis, maternally supplied cholesterol could, even though incompletely, support the embryonic growth by E9.5.

The phenotype of *Hmgcr* null mice is strikingly severer than that of *Ss*-/- mice. In addition to loss of *de novo* cholesterol synthesis, disruption of mevalonate synthesis has many ramifications, including loss of non-sterol isoprenoids essential for protein isoprenylation modifications and potential perturbation on *N*-linked glycosylation through inhibition of dolichol synthesis (1). Non-sterol isoprenoids serve as lipid

attachment for a variety of intracellular signaling molecules including small GTP-binding proteins, such as Rho, Ras, and Rac, whose proper membrane localization and function are dependent on isoprenylation (33). Given the role that these proteins play in pathways regulating cell survival, proliferation, differentiation, and cytoskeletal organization, it seems likely that the altered expression will manifest as markedly abnormal function during embryogenesis. Furthermore, studies with mice deficient in dolichol-mediated *N*-glycan formation suggest that *N*-glycosylation is also essential for the peri-implantation stage embryos (34). In addition, recent studies in *Drosophila*, a species without sterol synthesis, revealed another critical developmental function of HMG-CoA reductase in providing spatial information to guide migrating primordial germ cells (35). Indeed, HMG-CoA reductase is highly expressed during the development in humans (36), mice (37), sea urchin (38), and *Drosophila* (39). Studies with *in situ* hybridization revealed that HMG-CoA reductase mRNA is highly expressed in the post-imlantation rat embryos, from the egg cylinder stage through gastrulation, neurulation, and early organogenesis, with strong signal observed in primitive ectoderm and neural tube, which may reflect developmental requirements for products of the mevalonate pathway in these organs (30). In *Hmgcr*-/- mice, production of those non-sterol metabolites was hindered, while it may remained unaffected in *Ss*-/- mice. Therefore, perturbation of non-sterol isoprenoid synthesis would probably underlie the severer phenotypes of *Hmgcr*-/- embryos. Whether the lethal phenotype observed in the HMG-CoA reductase deficiency results from deficiency of some specific isoprenoids or from a more global deficiency of isoprenoids is uncertain at present.

A requirement for mevalonate-derived isoprenoids in early embryogenesis was reported two decades ago, in studies of cultured embryos where compactin, a classic HMG-CoA reductase inhibitor, interrupted their preimplantation development after 32-

cell stage *in vitro*, the effect which was reversed by mevalonate supplementation (37). Interestingly, ultrastructural examination of growth-arrested embryos revealed a predominance of nuclei with highly condensed chromatin, a hallmark of apoptosis. However, *in vivo* studies with various statins generally exhibited neither teratogenic nor embryotoxic effect when administered even at maternally toxic doses (40-43). Only early studies with mevinolin (lovastatin) showed that high doses of this compound administered to pregnant rats resulted in fetal malformations of the vertebrae and ribs, as well as gastroschisis (failure of abdominal wall to close) (44). Collectively, the effects of HMG-CoA reductase inhibition on embryogenesis had not been fully established. Our results, however, have clearly demonstrated that HMG-CoA reductase is crucial in very early steps of embryogenesis.

The reasons why mevalonate supplementation failed to completely rescue *Hmgcr-/-* pups are not clear. The fact that growth arrest of blastocysts by compactin and teratogenic effects of mevinolin were readily reversed by administered mevalonate indicates that they were due to end-product deficiency and not to precursor build-up. At mid and late gestation, mevalonate was demonstrated to cross the maternal-fetal barrier (45), suggesting that maternally derived mevalonate might be utilized by later-stage developing embryos. Although in early stage embryos mevalonate seems to be permeable into embryonic cells from intrauterine environment, cellular amount of mevalonate might be in tight control to support proper peri-implantation development, making its unregulated supplementation to the dams invalid. Alternatively, transfer of maternal mevalonate to the embryos might require proper development of cholioallantoic circulatory system.

Most of human malformation syndromes due to inborn errors of cholesterol biosynthesis involve enzymes at the distal steps of the mevalonate pathway, and not at

more proximal steps (Reviewed in Refs. 46,47), indicating that many of the intermediates of the cholesterol biosynthetic pathway are essential for fundamental cellular processes and the loss of these important pathways would result in cell death and early embryonic lethality. The absence of human genetic disease caused by HMG-CoA reductase deficiency suggests that the defect would cause prenatal death also in humans as observed in *Hmgcr*-null mice. In this regard, it is interesting to note that HMG-CoA reductase activity in the liver of human anencephalic fetus was reported to be 10-fold less than in the liver of normal fetus (48), although the causal relationship was not implied. On the other hand, several subjects with partial absence of mevalonate kinase, the enzyme immediately following HMG-CoA reductase, have been described (49). These patients are characterized by mevalonic aciduria and a broad range of phenotypic findings including malformations of the central nervous system, facial dysmorphy, psychomotor retardation, and recurrent fever episodes. Plasma cholesterol levels are normal in subjects with mevalonate kinase deficiency, suggesting that compensatory mechanisms, such as increased activities of HMG-CoA reductase and the LDL receptor pathway (50), are sufficient to overcome the enzyme deficiency and provide adequate synthesis of cholesterol as well as non-sterol intermediates for embryogenesis.

Several mutant cell lines deficient in HMG-CoA reductase have been characterized (51). One such cell line, designated as UT-2 cells, is a mutant clone of Chinese hamster ovary (CHO) cells that expresses only 2-5% of the HMG-CoA reductase activity of parental CHO cells and requires exogenous mevalonate for growth (52). Recently, Engfelt et al. identified the responsible mutations in the structural gene for reductase (16). By growing UT-2 cells in the absence of mevalonate, they have established a new cell line, designated as UT-2* cells, and demonstrated that UT-2*

cells had up-regulated HMG-CoA reductase activity which was localized exclusively to peroxisomes and the peroxisomal enzyme in these cells was sufficient for survival without mevalonate (15,17). Based on the detailed characterization of UT-2* cells, they suggested the existence of a second, peroxisome-specific gene for the reductase. Presence of multiple genes encoding HMG-CoA reductase in lower organisms such as yeast, fungi and plants also appeared to support the notion of a second reductase gene in mammals.

Contrary to these observations, the lethal phenotype of *Hmgcr*-/- mice argues against the presence of a second gene, irrespective of whether it encodes a peroxisomal or ER reductase, which could functionally replace the classical ER reductase, at least in the very early stage of development. Consistently, in a recent review Breitling and Krisans noted that comprehensive analysis of genome sequence of human and mouse through homology search revealed no evidence of a second gene in mammals (53). They concluded that the peroxisomal activity is due to alternative targeting of the ER enzyme to peroxisomes by an as yet uncharacterized mechanism. Establishment of *Hmgcr*-/- ES cell lines, if possible, would provide another opportunity to address these issues.

In *Hmgcr*+/− mice, the HMG-CoA reductase activities and rate of cholesterol biosynthesis in the liver remained unaffected, indicating that the haploid amount of *Hmgcr* gene is not rate-limiting in hepatic cholesterol homeostasis. This is not surprising in light of multivalent control of HMG-CoA reductase activities in order to retain cholesterol homeostasis. On the other hand, *Hmgcr*+/- MEFs exhibited 50% reduction in enzymatic activities in either basal or activated condition examined, suggesting that HMG-CoA reductase activities in MEFs may simply reflect the gene dosage, while that in the liver is controlled by a more complex mechanism.

In conclusion, we have generated a murine model of HMG-CoA reductase deficiency, which should provide the basis for understanding the roles of mevalonate pathway in the embryonic development as well as the pleiotropic effects of statins.

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FOOTNOTES

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[1]The abbreviations used are: HMG-CoA, 3-hydroxy-3-methyglutaryl-coenzyme A; ER, endoplasmic reticulum; E, embryonic day; MEF, mouse embryonic fibroblast; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; LDL, low density lipoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; CHO, Chinese hamster ovary.

FIGURE LEGENDS

Fig. 1. Targeted disruption of the *Hmgcr* gene. *A*, a map of the *Hmgcr* locus, together with the sequence replacement gene-targeting vector and the targeted *Hmgcr* allele. Only relevant exons (closed boxes) and introns (thick lines) are shown. The gene targeting event replaced the region spanning exon 12-14 with a cassette containing a neomycin resistance gene (*neo*) driven by the RNA polymerase II promoter. The transcriptional direction of the *neo* gene is indicated by the arrow in its box. Two copies of herpes simplex virus thymidine kinase (*HSV-TK*) expression cassettes were used as a negative selection marker. The location of the 0.5-kb probe used for Southern blot analysis is denoted by a closed bar. Upon homologous recombination, disrupted allele yields a 8.7-kb *Eco*RI fragment, whereas the corresponding fragment for wild-type allele is 16 kb. E, *Eco*RI; P1, primer 1; P2, primer 2; kb, kilobase pairs. *B*, Southern blot analysis of *Eco*RI-digested tail DNA from F_1 offspring to confirm germ line transmission of the targeted allele. The positions of migration of the *Eco*RI fragments derived from wild-type and disrupted alleles are indicated. Molecular size markers are shown on the right.

Fig. 2. Genotyping of blastocyst. *A*, Scheme for PCR detection of wild-type and disrupted *Hmgcr* alleles. Exon 14 (solid box), the *neo*-expression cassette (open box), and the location of primers 1, 3 and 4 are depicted. *B*, Representative Southern blot analysis of PCR products amplified from blastocyst DNA. Wild-type (300 bp) and mutant (220 bp) bands are indicated.

Fig. 3. Hepatic expression of the genes involved in cholesterol homeostasis in *Hmgcr*+/− mice. Northern blot for HMG-CoA reductase, HMG-CoA synthase,

farnesyl diphosphate synthase, squalene synthase, LDL receptor (upper panels), and GAPDH (lower panels) as a loading control using liver poly(A^+) RNA. W, wild-type; H, heterozygote.

Fig. 4. Activity of HMG-CoA reductase in cellular extracts from MEFs. MEFs were grown to 60-70% confluence in 10% FCS-DMEM. After the monolayers were cultured in DMEM containing either 10% FCS, or 5 mg/ml LPDS with or without 100 nM simvastatin for 6 h, the cells were detergent-solubilized and the extracts were assayed for HMG-CoA reductase activity. Simvastatin was diluted out before assay to ensure full activity measurements. Each value represents the average of 9 *Hmgcr*+/- or 6 *Hmgcr*+/+ MEF clones +/- S.D. *, $p < 0.005$; **, $p < 0.001$.

Table I: Genotypes of offspring from intercrosses of *Hmgcr*+/- mice.

	Genotype				
Age	+/+	+/−	−/−	Resorbed	Total
3 weeks	52	106	0	NA	158
E13.5	3	7	0	0	10
E8.5-9.5	17	26	0	0	43
E3.5	13	42	19	NA	74

NA, not applicable.

Table II: Effects of maternal mevalonate supplementation on the genotypes of offspring from heterozygous intercrosses.

Age	Appearance		Genotype			Total
	Normal	Abnormal*	+/+	+/-	-/-	
3 weeks	31	0	10	21	0	31
E9.5-10.5	39	10	16	23	0	49

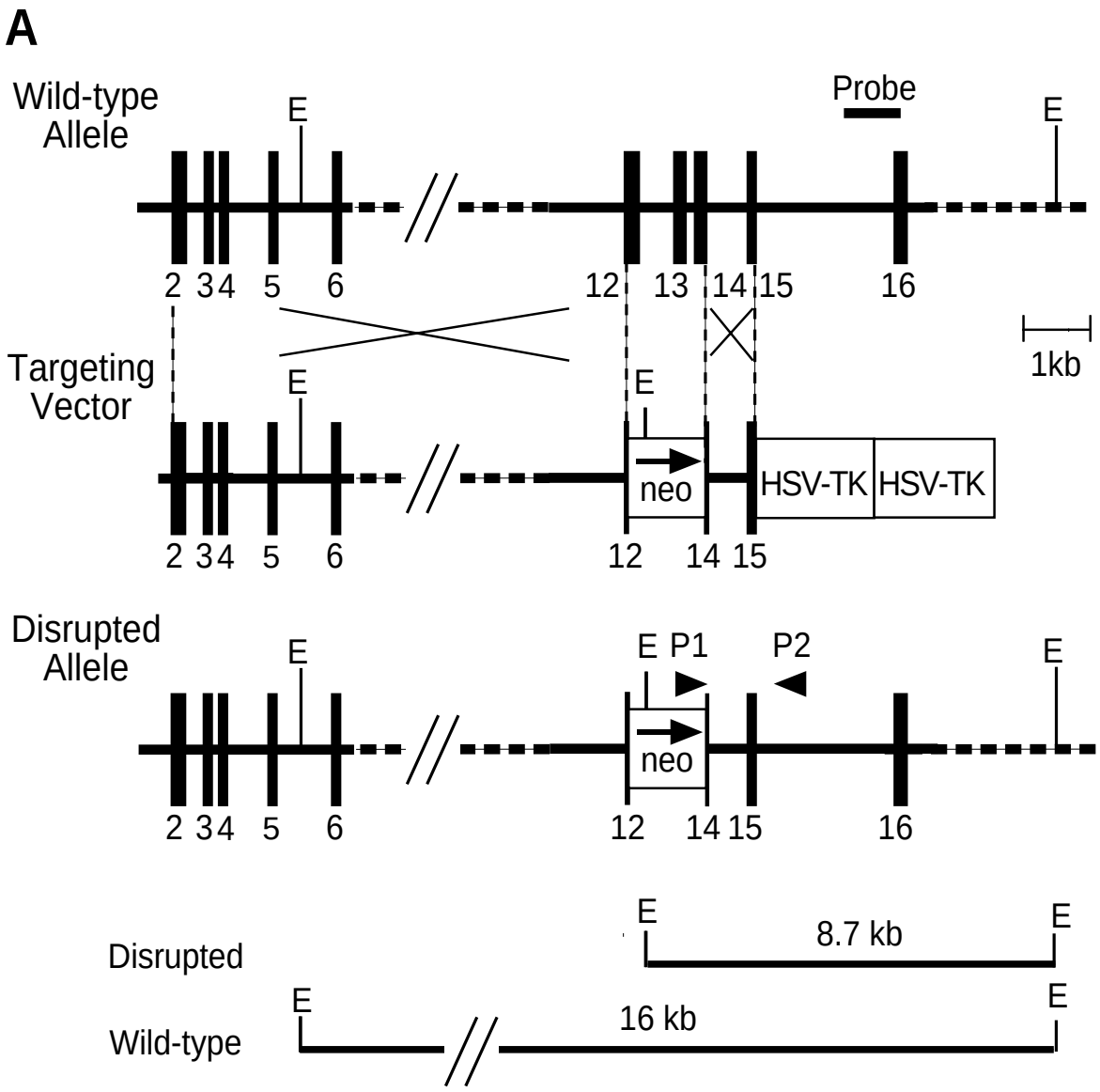
* Abnormal embryos were with egg cylinder stage appearance at E9.5-10.5 and their precise genotyping was not successful.

Table III: Phenotypic comparison of *Hmgcr*+/+ and *Hmgcr*+/- mice.

Parameter	Number of mice	Genotype	
		Wild-type	Heterozygote
HMG-CoA reductase activity (pmol/mg protein/min)	5	129.6±78.7	117.5±59.3
[^{14}C]Acetate incorporation into sterols (dpm/100 mg liver/h)	6	1217.8±793.4	1008.3±436.3
Liver cholesterol content (mg/g)	5	3.5±0.47	3.0±0.44
Liver triglyceride content (mg/g)	5	15.2±2.2	11.6±6.3
Plasma total cholesterol (mg/dl)	14	94.0±19.4	100.5±30.9
Plasma triglycerides (mg/dl)	20	82.3±24.7	84.3±35.3

Each value represents mean +/- S.D. of the indicated number of 8-12-week-old littermate mice of the indicated genotype. All mice were fed a normal chow diet. For determination of plasma lipid levels, blood was collected after a 4-h fast. No significant differences were noted between *Hmgcr*+/+ and *Hmgcr*+/- mice for each parameter.

Fig. 1


A
Wild-type Allele
E
Probe
E
2 3 4 5 6
12 13 14 15
16
1kb
Targeting Vector
E
E
neo
HSV-TK
HSV-TK
2 3 4 5 6
12 14 15
Disrupted Allele
E
E P1
P2
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E
16 kb
E

B

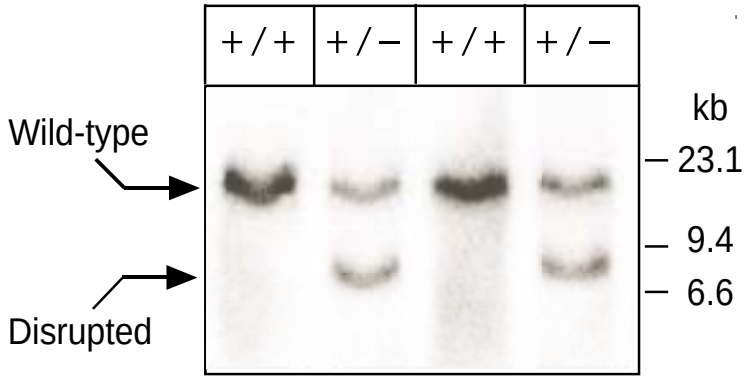

+/+
+/−
+/+
+/−
kb
Wild-type
23.1
9.4
6.6
Disrupted

Fig. 2

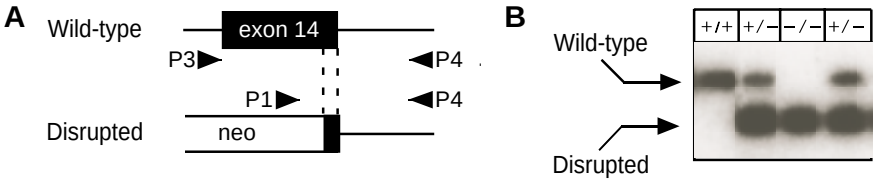

A
Wild-type
exon 14
P3
P4
P1
P4
Disrupted
neo
B
Wild-type
Disrupted
+/+
+/−
−/−
+/−

Fig. 3

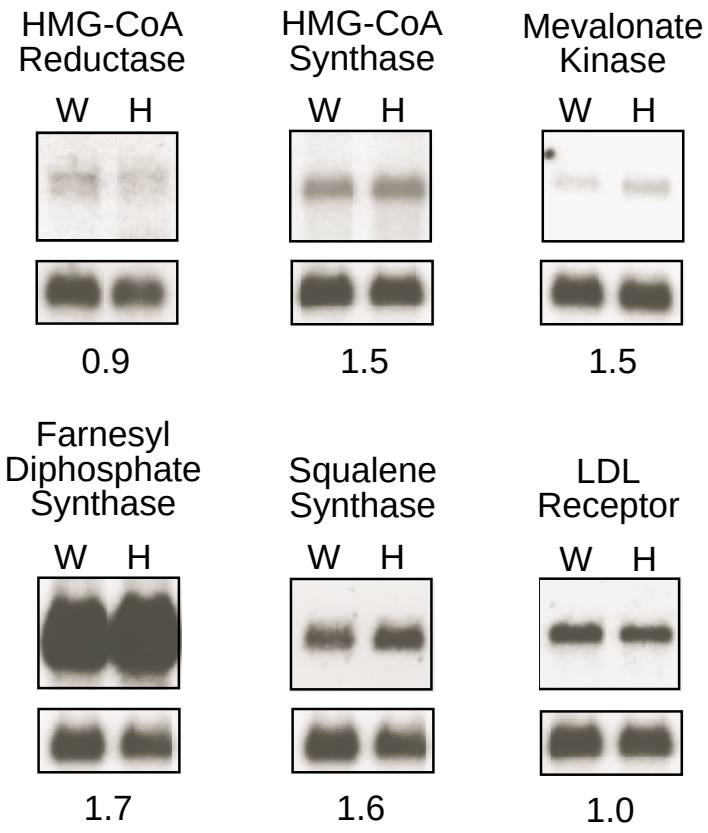
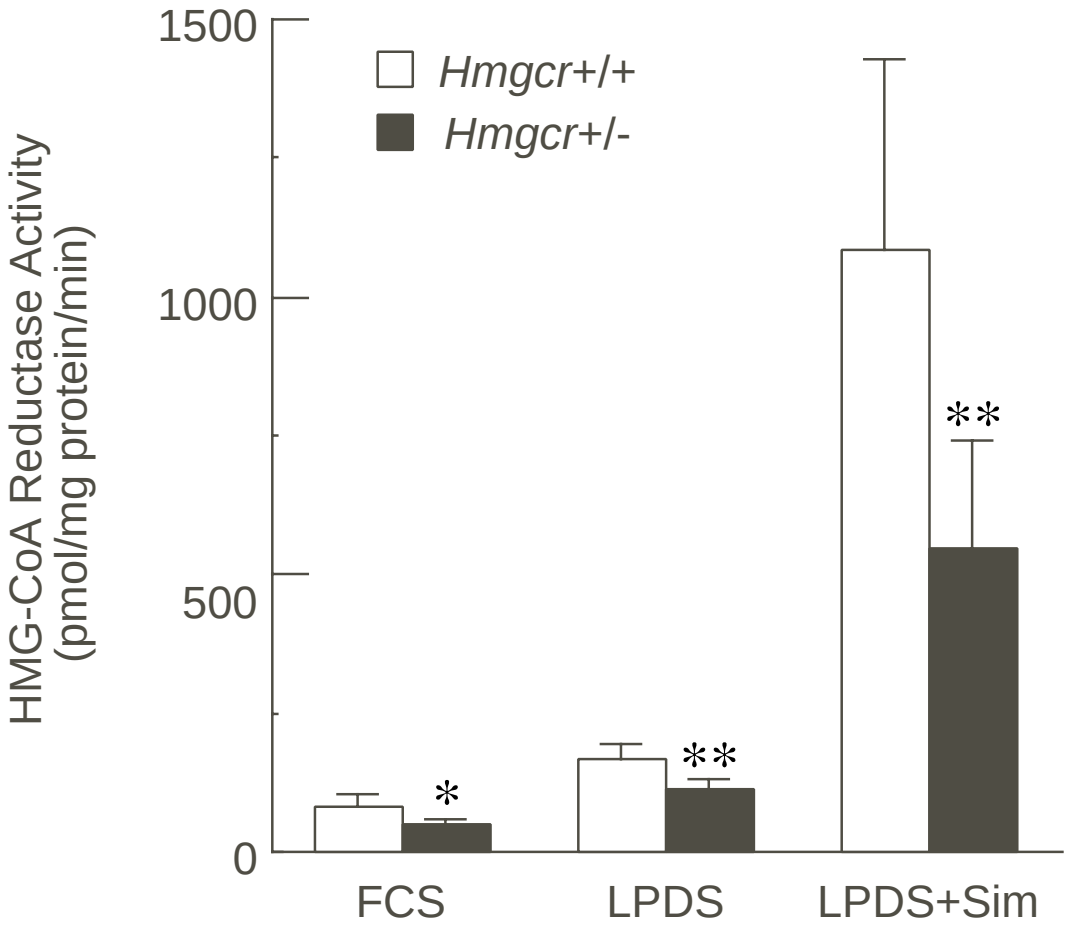

HMG-CoA Reductase
W H
0.9
HMG-CoA Synthase
W H
1.5
Mevalonate Kinase
W H
1.5
Farnesyl Diphosphate Synthase
W H
1.7
Squalene Synthase
W H
1.6
LDL Receptor
W H
1.0

Fig. 4


HMG-CoA Reductase Activity
(pmol/mg protein/min)
1500
1000
500
0
Hmgcr+/+
Hmgcr+/-
*
**
**
FCS
LPDS
LPDS+Sim