Endogenous sterol intermediates of the mevalonate pathway regulate HMGCR degradation and SREBP-2 processing

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Abstract Sterol-regulated HMG-CoA reductase (HMGCR) degradation and SREBP-2 cleavage are two major feedback regulatory mechanisms governing cholesterol biosynthesis. Reportedly, lanosterol selectively stimulates HMGCR degradation, and cholesterol is a specific regulator of SREBP-2 cleavage. However, it is unclear whether other endogenously generated sterols regulate these events. Here, we investigated the sterol intermediates from the mevalonate pathway of cholesterol biosynthesis using a CRISPR/Cas9-mediated genetic engineering approach. With a constructed HeLa cell line expressing the mevalonate transporter, we individually deleted genes encoding major enzymes in the mevalonate pathway, used lipidomics to measure sterol intermediates, and examined HMGCR and SREBP-2 statuses. We found that the C4-dimethylated sterol intermediates, including lanosterol, 24,25-dihydrolanosterol, follicular fluid meiosis activating sterol, testis meiosis activating sterol, and dihydro-testis meiosis activating sterol, were significantly upregulated upon mevalonate loading. These intermediates augmented both degradation of HMGCR and inhibition of SREBP-2 cleavage. The accumulated lanosterol induced rapid degradation of HMGCR, but did not inhibit SREBP-2 cleavage. The newly synthesized cholesterol from the mevalonate pathway is dispensable for inhibiting SREBP-2 cleavage. Together, these results suggest that lanosterol is a bona fide endogenous regulator that specifically promotes HMGCR degradation, and that other C4-dimethylated sterol intermediates may regulate both HMGCR degradation and SREBP-2 cleavage. —Chen, L., M-Y. Ma, M. Sun, L-Y. Jiang, X-T. Zhao, X-X. Fang, S. M., Lam, G-H. Shui, J. Luo, X-J. Shi, and B-L. Song. Endogenous sterol intermediates of the mevalonate pathway regulate HMGCR degradation and SREBP-2 processing. J. Lipid Res. 2019. 60: 1765–1775.

Cholesterol is an essential lipid for mammals. It regulates membrane fluidity and functions, serves as the precursor for steroid hormones and bile acids, and covalently modifies the Hedgehog and Smoothened proteins (1–3). A high level of blood cholesterol is a major risk factor for cardiovascular disease, and cholesterol lowering is an effective way to treat the cardiovascular disease (4–7).

Cholesterol is synthesized from acetyl-CoA through over 30 steps of reactions (Fig. 1). The cholesterol biosynthetic pathway is also known as the mevalonate pathway. Mevalonate is a key intermediate synthesized from HMG-CoA by HMG-CoA reductase (HMGCR), an ER-localized rate-limiting enzyme of the mevalonate pathway (8). Cholesterol biosynthesis is governed by two feedback regulatory mechanisms: the sterol-induced degradation of HMGCR (9) and inactivation of SREBP-2, the latter of which controls transcription of the genes involved in cholesterol biosynthesis and uptake (10). When the cellular sterol level is

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Abbreviations: CYP51A1, cytochrome P450 family 51 subfamily A member 1; DHCR24, 24-dehydrocholesterol reductase; 24,25-DHL, 24,25-dihydrolanosterol; DKO, double KO; FDFT1, farnesyl diphosphate farnesyltransferase 1; FF-MAS, follicular fluid meiosis activating sterol; 25-HC, 25-hydroxycholesterol; HeLa/MT, HeLa cells stably expressing the mevalonate transporter; HMGCR, HMG-CoA reductase; MCT1, monocarboxylate transporter 1; MCT1<sup>ΔαMC</sup>, monocarboxylate transporter 1 with a phenylalanine-to-cysteine substitution at the amino acid site 360; MSMO1, methylsterol monooxygenase 1; MT, mevalonate transporter; PC, phosphatidylcholine; SCAP, SREBP cleavage activating protein; T-MAS, testis meiosis activating sterol.

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high, HMGCR binds to the E3 ubiquitin ligase gp78, TRC8, or RNF145, catalyzing ubiquitination and proteasomal degradation of HMGCR (11–15). High levels of cholesterol also promote the SREBP cleavage activating protein (SCAP)-SREBP-2 complex to bind the ER retention protein Insig, preventing proteolytic activation of SREBP-2 (16). These two sterol-regulated pathways together with cholesterol-induced degradation of squalene monooxygenase (17) primarily downregulate cholesterol biosynthesis at high sterol levels.

In addition to cholesterol, the mevalonate pathway produces dozens of sterol intermediates and other nonsterol products, including heme, coenzyme Q, dolichol, farnesyl, and geranylgeranyl (Fig. 1). Some metabolites in the mevalonate pathway are important signal molecules (1, 2, 17–22). For example, cholesterol is a specific regulator of SREBP-2. It binds and induces the conformational change of SCAP, blocking the processing of SREBP-2 (19, 23, 24). However, cholesterol does not stimulate HMGCR ubiquitination and degradation (18). Lanosterol, on the other hand, promotes degradation of HMGCR but fails to inhibit SREBP-2 processing (18). The oxidative derivatives of cholesterol, such as 25-hydroxycholesterol (25-HC), can both stimulate HMGCR degradation and inhibit SREBP-2 processing, probably through binding Insig proteins (25).

In previous studies, the regulation of HMGCR degradation and SREBP-2 processing were mainly analyzed using in vitro assays or cultured cells exposed to different sterols (26), with conflicting conclusions being reported. We found that exogenously added lanosterol, 24,25-dihydroxylanosterol (24,25-DHL), or other 4,4-dimethylated sterols could stimulate HMGCR ubiquitination in vitro and HMGCR degradation in cultured cells (18). Recently, we developed a lanosterol derivative (named HMG499 or compound 81) that effectively degrades HMGCR in cultured cells and lowers diet-induced hypercholesterolemia in mice (27). Consistently, Nguyen et al. (28) observed HMGCR degradation under hypoxia, a condition that causes lanosterol and 24,25-DHL accumulation because oxygens are indispensable for converting lanosterol to cholesterol. However, Lange et al. (29) reported that 24,25-DHL, rather than lanosterol, inhibited HMGCR activity when added in the culture medium with different experimental procedures. Given the unavailability of potent inhibitors for each enzyme and incompetency of RNAi to completely abolish an enzyme’s function, it is still elusive which endogenously generated products in the mevalonate pathway regulate HMGCR degradation.

In this study, we generated a HeLa cell line stably expressing the mevalonate transporter (MT) that efficiently takes up mevalonate when it is present in high concentration in the medium. We then used CRISPR/Cas9 technique to individually knock out genes encoding each enzyme of the mevalonate pathway and measured the intermediates using lipidomics. By this method, we can completely shut down the mevalonate pathway at any desired step and control the intermediate(s) accumulation by adding mevalonate. We found that KO of farnesyl diphosphate farnesyltransferase 1 (FDFT1) completely abolished mevalonate-regulated HMGCR degradation and SREBP-2 cleavage. KO of both cytochrome P450 family 51 subfamily A member 1 (CYP51A1) and 24-dehydrocholesterol reductase (DHCR24) caused accumulation of lanosterol that specifically promoted HMGCR degradation without inhibiting
SREBP-2 cleavage. The C4-dimethylated sterol intermediates, which were elevated by ablation of methylsterol monooxygenase 1 (MSMO1), showed marked potency in triggering HMGCR degradation and SREBP-2 cleavage. In summary, the study identifies that lanosterol specifically promotes HMGCR degradation, and other C4-dimethylated sterol intermediates may regulate both HMGCR degradation and SREBP-2 cleavage.

MATERIALS AND METHODS

Reagents and antibodies

We obtained lovastatin, mevalonate, and 25-HC from Sigma. Lipoprotein-deficient serum was prepared from newborn calf serum as described previously (30). The primary antibodies were as follows: mouse monoclonal antibody (clone A9) against HMGCR (amino acids 450–887) (31); mouse monoclonal antibody (clone 1D2) against the NH2 terminus of human SREBP-2 (amino acids 48–403) (32, 33); mouse monoclonal antibody (clone AC-15) against β-ACTIN (A1978; Sigma); rabbit polyclonal antibody against GAPDH (10494-1-AP; Proteintech); rabbit monoclonal antibody against CYP51A1 (13431-1-AP; Proteintech); mouse monoclonal antibody (clone AC-15) against β-ACTIN (A1978; Sigma); rabbit polyclonal antibody against HMGCR degradation and SREBP-2 cleavage.

Generation of HeLa/MT cells

The sequences of the MT, monocarboxylate transporter 1 (MCT1) with a phenylalanine-to-cysteine substitution at the amino acid site 360 (MCT1F360C), have been described previously (34). The MCT1F360C expression cassette was cloned into the pLVX-IRES-Puro vector fused with a Flag tag. The HeLa cell stably expressing MCT1F360C was generated and named as HeLa/MT.

Generation and identification of KO cell line

The KO cell line was generated by CRISPR/Cas9 technique as previously described (35). Briefly, lentivirus CRISPR (one-vector, lentiviral GeCKO system) from the Feng Zhang laboratory was used to express SRNA and Cas9. HeLa/MT cells were transfected with plasmids harboring SRNA sequence and screened with puromycin. The KO cell line was selected and identified via DNA sequencing or Western blotting. The SRNA sequences were as follows: FDBT1, GACTGCGGGTTCACTGAGGAC; CYP51A1, TAATCCTGATGCGTACTTAC; MSI01, GTGCAGTCATTGAAGATACT; DHR24, AGGTGTACGACAAGATCTGC.

Sterol extraction and lipidomics analysis

HeLa and HeLa/MT cells were set up at a density of 2.5 × 10^6 cells per 100 mm dish (two dishes per sample) in DMEM medium supplemented with 10% FBS on day 0. On day 1, the cells were washed twice with PBS and then switched to medium A (DMEM, 5% lipoprotein-deficient serum, and 1 μM of lovastatin) for 16 h at 37°C. On day 2, for concentration-dependent assays, the cells were switched to medium A supplemented with the indicated concentrations of lovastatin or 25-HC for 4 h. For time-course assays, the cells were switched to medium A supplemented with 3 mM mevalonate for the indicated time. The cells were collected, subjected to SDS-PAGE, and transferred to PVDF membranes. Immunoblot analysis was carried out using monoclonal IgG-A9 (against HMGCR) and other antibodies. All the experiments were independently repeated three or more times. Western blotting signal was acquired by autoradiography machine (Tanon-5200). In some experiments, the HMGCR blots were spliced to obtain a similar baseline of protein levels. Quantification of the HMGCR protein level was performed by ImageJ (National Institutes of Health, https://imagej.nih.gov/ij/) and normalized with β-ACTIN or GAPDH.

Immunoblotting and quantification of SREBP-2

HeLa or HeLa/MT cells were set up at a density of 4 × 10^5 cells per 60 mm dish (two dishes per sample) in DMEM medium supplemented with 10% FBS on day 0. On day 1, the cells were washed twice with PBS and then depleted with medium B (DMEM, 5% lipoprotein-deficient serum, and 1 μM of lovastatin) for 4 h. On day 2, for concentration-dependent assays, the cells were washed twice with PBS and then switched to medium A supplemented with the indicated concentrations of mevalonate or 25-HC for 4 h. For time-course assays, the cells were washed twice with PBS and then switched to medium A supplemented with 3 mM mevalonate medium for the indicated time. Medium A was added with 25 μg/ml of N-acetyl-leucinal-leucinal-norleucinal and 5 μM of MG132 1.5 h prior to harvesting cells. The cells were collected and subjected to SDS-PAGE, transferred to PVDF membranes, and then immunoblot analysis was carried out using monoclonal IgG-1D2 (against lanosterol specifically promotes HMGCR degradation 1767
SREBP-2). All the experiments were repeated three times. Western blotting signal was acquired via autoradiography machine (Tanon-5200). In some experiments, the SREBP-2 blots were spliced to obtain a similar baseline of protein levels. Quantification of SREBP-2 was performed by ImageJ; the SREBP-2 processing level was evaluated by the nuclear form normalized with the sum of precursor form and nuclear form \([n/(n+p)]\).

RESULTS

Regulation of HMGCR degradation and SREBP-2 cleavage in HeLa/MT cells

We first sought to establish a system to evaluate HMGCR degradation and SREBP-2 cleavage in response to endogenous regulators generated from mevalonate. Normally cultured cells usually take up mevalonate with low efficiency (13). To increase the metabolic flux and augment the regulatory effects of mevalonate on HMGCR and SREBP-2, we generated a cell line stably expressing the monocarboxylate transporter 1 (MCT1) with a phenylalanine-to-cysteine substitution at the amino acid site 360 (MCT1F360C), which allows for efficient uptake of mevalonate into cells (34, 38). The stable cell line was named HeLa/MT.

Next, we performed a lipidomics study to measure the levels of the intermediates starting from squalene to cholesterol in both HeLa and HeLa/MT cells incubated with or without 3 mM of mevalonate (Table 1). Cholesterol was the most abundant, accounting for >99% of the tested sterols. In HeLa cells without mevalonate treatment, the level of cholesterol was approximately 1,000- to 10,000-fold that of other sterol intermediates. Almost all tested sterol intermediates were dramatically increased in HeLa/MT cells compared with those in HeLa cells, especially following mevalonate treatment. In particular, lanosterol, 24,25-DHL, follicular fluid meiosis activating sterol (FF-MAS), testis meiosis activating sterol (T-MAS), and dihydro-T-MAS in HeLa/MT cells were increased by 61.5-, 31.6-, 71.6-, 88.4-, and 65-fold, respectively (Fig. 2A, Table 1), suggesting that the intermediates from squalene to cholesterol were still regulated when the Kandutsch-Russell pathway or the DHCR24-Russell pathways. We then knocked out \(FDFT1\) to determine whether HMGCR degradation and SREBP-2 cleavage in \(FDFT1\)-deficient cell lines (Fig. 3B–C). These results suggest that the intermediates from squalene to cholesterol are required for regulating HMGCR degradation and SREBP-2 cleavage.

Deletion of \(FDFT1\) abolishes mevalonate-mediated negative feedback regulation

We next knocked out the enzymes involved in the metabolic flux using the CRISPR/Cas9 technique and tested the effects of accumulating intermediates on HMGCR degradation and SREBP-2 cleavage in HeLa/MT cells. FDFT1, also named squalene synthase, catalyzes the first committed step of sterol synthesis by converting two farnesyl diphasphate molecules to squalene (Fig. 1). In \(FDFT1\)-deficient HeLa/MT cells, squalene and its downstream products were all dramatically decreased even when 3 mM of mevalonate was added (Fig. 3A, Table 2). Notably, mevalonate failed to induce HMGCR degradation or suppress SREBP-2 cleavage in \(FDFT1\)-deficient cells (Fig. 3B–E, supplemental Fig. S1). As control, 25-HC could similarly promote HMGCR degradation in WT and \(FDFT1\)-deficient cell lines (Fig. 3B–C). These results suggest that the intermediates from squalene to cholesterol are required for regulating HMGCR degradation and SREBP-2 cleavage.

The sterol intermediates in the Bloch pathway are sufficient for feedback regulation

Starting from lanosterol, metabolic flux is separated into two parallel pathways called the Bloch and Kandutsch-Russell pathways. We then knocked out \(DHCR24\) to determine whether HMGCR degradation and SREBP-2 cleavage were still regulated when the Kandutsch-Russell pathway was abolished and no cholesterol was newly synthesized.

### Table 1. Lipidomic analysis of HeLa and HeLa/MT cells

| Parameters         | HeLa Cell Without Mevalonate | HeLa Cell With Mevalonate (3 mM) | HeLa/MT Cell Without Mevalonate | HeLa/MT Cell With Mevalonate (3 mM) |
|--------------------|------------------------------|----------------------------------|---------------------------------|-------------------------------------|
|                    | Absolute Value [nmol/PC (μmol)] |                                   |                                 |                                     |
| Squalene           | 0.0027 ± 0.00052             | 0.0083 ± 0.0048                  | 0.0063 ± 0.0019                 | 0.026 ± 0.0051                      |
| Lanosterol         | 0.08 ± 0.03                  | 2.21 ± 2.54                     | 0.10 ± 0.03                     | 6.15 ± 0.68                        |
| 24,25-DHL          | 0.05 ± 0.03                  | 0.14 ± 0.02                     | 0.20 ± 0.05                     | 6.32 ± 1.24                        |
| FF-MAS             | 0.02 ± 0.00                  | 0.74 ± 0.16                     | 0.05 ± 0.01                     | 3.58 ± 0.75                        |
| T-MAS              | 0.12 ± 0.01                  | 3.75 ± 2.50                     | 0.20 ± 0.03                     | 17.68 ± 1.75                       |
| Dihydro-T-MAS      | 0.34 ± 0.32                  | 2.26 ± 0.55                     | 0.25 ± 0.02                     | 16.24 ± 1.00                       |
| Zymosterol         | 0.04 ± 0.01                  | 0.56 ± 0.31                     | 0.10 ± 0.01                     | 1.06 ± 0.19                        |
| Dehydrocholesterol | 0.14 ± 0.08                  | 0.20 ± 0.01                     | 0.39 ± 0.27                     | 29.50 ± 6.45                       |
| Lathosterol        | 0.90 ± 0.40                  | 3.32 ± 2.16                     | 1.44 ± 0.27                     | 8.45 ± 3.39                        |
| 7-Dehydrocholesterol | 1.07 ± 0.74                 | 3.00 ± 0.77                     | 2.20 ± 1.31                     | 15.97 ± 4.53                       |
| Desmosterol        | 0.62 ± 0.08                  | 5.51 ± 3.22                     | 0.30 ± 0.06                     | 1.92 ± 0.53                        |
| Cholesterol        | 980 ± 160                    | 1640 ± 1160                     | 1240 ± 260                      | 1420 ± 480                         |

HeLa cells and HeLa/MT cells were depleted of sterol in medium A for 16 h, and then switched to medium A supplemented with or without 3 mM mevalonate for 3 h. The cells were collected and subjected to lipidomics analysis. The level of various sterol intermediates is shown as absolute level normalized with micromoles of PC ± SEM (n = 3).
Lanosterol specifically promotes HMGCR degradation

Figure 4A and Table 2 show that all tested intermediates in the Kandutsch-Russell pathway, including 24,25-DHL, dihydro-T-MAS, lathosterol, and 7-dehydrocholesterol, were dramatically decreased even in the presence of mevalonate. By contrast, the sterols in the Bloch pathway, including lanosterol, FF-MAS, T-MAS, zymosterol, dehydrosterol, and desmosterol, were all substantially increased. Interestingly, mevalonate could still promote HMGCR degradation (Fig. 4B, C) and inhibit SREBP-2 cleavage potently (Fig. 4D, E) in DHCR24-deficient cells. These results demonstrate that the sterol intermediates in the Bloch pathway are sufficient for regulating HMGCR degradation and SREBP-2 cleavage.

Lanosterol can regulate HMGCR degradation but not SREBP-2 cleavage

Next, we generated CYP51A1 KO and CYP51A1-DHCR24 double-KO (DKO) HeLa/MT cells. Lipidomics analysis showed that lanosterol and 24,25-DHL were dramatically increased and other downstream sterols were decreased in CYP51A1 KO cells. Only lanosterol was substantially increased in CYP51A1-DHCR24 DKO cells, while all tested downstream intermediates, including FF-MAS, dihydro-T-MAS, zymosterol, lathosterol, and desmosterol, were largely reduced (Fig. 5A, Table 3). Notably, mevalonate was able to promote HMGCR degradation in WT, CYP51A1 KO, and CYP51A1-DHCR24 DKO HeLa/MT cells in a concentration-dependent manner (Fig. 5B, C; supplemental Fig. S2A, B). The CYP51A1 KO and CYP51A1-DHCR24 DKO cells seemed to be more sensitive to mevalonate than WT cells. HMGCR degradation was more rapid in CYP51A1 KO and CYP51A1-DHCR24 DKO cells than in WT cells (supplemental Fig. S2C and D). To rule out the possibility that increased geranylgeranyl pyrophosphate or other isoprenoids in CYP51A1-DHCR24 DKO cells may accelerate HMGCR degradation, we treated the cells with NB-598, an inhibitor of squa- lene monooxygenase. We found that NB-598 almost completely blocked mevalonate-induced HMGCR degradation in the WT and CYP51A1-DHCR24 DKO cells (supplemental Fig. S2E).

Next, we tested the SREBP-2 cleavage induced by varying concentrations of mevalonate (1.0–10 mM) (Fig. 5B, E) and different incubation times (2–6 h) (supplemental Fig. S3A, B) in these three cell lines. KO of both CYP51A1 and DHCR24 but not of CYP51A1 alone in HeLa/MT cells completely abolished the inhibition of SREBP-2 cleavage by mevalonate (Fig. 5D, E; supplemental Fig. S3A, B). As a control, 25-HC inhibited SREBP-2 cleavage in WT, CYP51A1 KO, and CYP51A1-DHCR24 DKO cells with similar potency (supplemental Fig. S3C, D). Together, these results demonstrate that lanosterol is an endogenous regulator specifically promoting HMGCR degradation without inhibiting SREBP-2 cleavage.
C4-dimethylated sterol intermediates can regulate HMGCR degradation and SREBP-2 cleavage

We further knocked out MSMO1 in WT or DHCR24-deficient HeLa/MT cells where metabolic flux after T-MAS and dihydro-T-MAS was blocked. Genome DNA sequencing of MSMO1 KO cells revealed a frameshift mutation of MSMO1 that caused a premature stop at amino acid 157 (supplemental Fig. S4A). The C4-dimethylated sterol intermediates, including lanosterol, 24,25-DHL, FF-MAS, T-MAS, and dihydro-T-MAS, were increased by 43-, 31.3-, 25.4-, 4.9-, and 1.7-fold, respectively, in MSMO1 KO cells incubated with 3 mM of mevalonate compared with those without mevalonate treatment (Fig. 6A, Table 3). In MSMO1-DHCR24 DKO cells incubated with 3 mM of mevalonate,
The sterol intermediates in the Bloch pathway are sufficient to induce feedback regulation. A: WT and DHCR24-deficient HeLa/MT cells were depleted of sterol in medium A for 16 h, and then switched to medium A supplemented with or without 3 mM of mevalonate for 5 h. The cells were collected and subjected to lipidomics analysis. The levels of each sterol intermediate were normalized to those in WT cells without mevalonate treatment. The absolute levels of various sterol intermediates are shown in Table 2. B: The cells were depleted of sterol by incubating in medium A for 16 h and refed with medium A containing the indicated concentrations of mevalonate or 25-HC for 5 h. The cells were then lysed, and Western blotting was carried out with monoclonal anti-HMGCR (IgG-A9), anti-DHCR24, and anti-GAPDH. C: Quantification of HMGCR in B. The HMGCR protein level was quantified by ImageJ and normalized to GAPDH. The HMGCR levels in WT or DHCR24-deficient HeLa/MT cells without treatment were defined as 1. D: The cells were depleted of sterol by incubating in medium B for 16 h, and then switched to medium A supplemented with the indicated concentrations of mevalonate or 25-HC for 4 h. The cells were...
lanosterol, FF-MAS, and T-MAS were increased by approximately 249.8-, 51.9-, and 1.7-fold, respectively. The downstream intermediates, including zymosterol, lathosterol, and desmosterol, were largely reduced in both MSMO1 KO and MSMO1-DHCR24 DKO cells (Fig. 6A).

The three cell lines were challenged by mevalonate with different concentrations (0.3–3.0 mM) (Fig. 6B, C; supplemental Fig. S4B, C) and incubation periods (2–6 h) (supplemental Fig. S4D, E). The mevalonate-induced degradation of HMGCR was dramatically accelerated in MSMO1 KO or MSMO1-DHCR24 DKO cells. The mevalonate-inhibited SREBP-2 cleavage seemed indistinguishable among the three cell lines (Fig. 6D, E; supplemental Fig. S4F, G). The above data indicate that the C4-dimethylated sterol intermediates, except for lanosterol, may both accelerate HMGCR degradation and inhibit SREBP-2 cleavage.

**DISCUSSION**

The mevalonate pathway is a conserved metabolic pathway that generates not only nonsterol products but also sterol intermediates and cholesterol (Fig. 1). Some of the products are signal molecules. Geranylgeranyl pyrophosphate and farnesyl diphosphate can be used to covalently modify proteins, a process called protein prenylation (39). T-MAS and FF-MAS are meiosis-activating sterols regulating the maturation of male and female germ cells, with the roles still controversial (40, 41). Desmosterol can activate LXR and inhibit the SREBP-2 pathway (42, 43). Cholesterol is a specific regulator of SREBP-2 processing and can activate the Hedgehog pathway via covalent modification of Hedgehog and Smoothened proteins (1, 2, 24). Lanosterol is the first cyclized sterol intermediate that specifically promotes the degradation of HMGCR (18), as confirmed by the current study. In addition, our results here suggest that other C4-dimethylated sterol intermediates can regulate both HMGCR degradation and SREBP-2 cleavage.

**Fig. 5.** Lanosterol specifically induces HMGCR degradation, but has no effect on SREBP-2 cleavage. A: WT, CYP51A1 KO and CYP51A1-DHCR24 DKO HeLa/MT cells were depleted of sterol by incubating in medium A for 16 h, and then switched to medium A supplemented with or without 3 mM of mevalonate for 5 h. The cells were lysed, and Western blotting was carried out with monoclonal anti-HMGCR (IgG-A9), anti-CYP51A1, anti-DHCR24, and anti-GAPDH. C: Quantification of HMGCR in (B). The HMGCR protein level was quantified by ImageJ and normalized to GAPDH. The HMGCR levels in WT, CYP51A1 KO or CYP51A1-DHCR24 DKO HeLa/MT cells without treatment were defined as 1. D: The cells were depleted of sterol by incubating in medium B for 16 h, and then switched to medium A supplemented with the indicated concentrations of mevalonate for 4 h. The cells were lysed, and Western blotting was carried out with monoclonal anti-SREBP-2 (IgG-1D2), pSREBP-2, precursor of SREBP-2; nSREBP-2, nuclear form of SREBP-2. E: Quantification of SREBP-2 in D. The nuclear and precursor forms of SREBP-2 were quantified by ImageJ and the ratio of nSREBP-2 to total SREBP-2 [n/(n + p)] was calculated. The ratios in lanes 1, 5, and 9 were defined as 1, respectively.
Lanosterol specifically promotes HMGCR degradation.

MCT1 is a monocarboxylate transporter for pyruvate and lactate, and the F360C is a gain-of-function mutation that enables MCT1 to transport mevalonate into the cell (34, 44, 45). The HeLa/MT cell line allowed us to manipulate the rate of metabolic flow by adding different concentrations of mevalonate into the culture medium. The CRISPR/Cas9 gene editing technique is a powerful tool to inactivate a specific gene and block the metabolic flux at any desired step. A combination of these two methods would be useful to study LXR, meiosis, G protein-coupled receptors (including Smoothened), and other biological events regulated by mevalonate metabolism.

Several criteria should be met when a metabolite is defined as a signal molecule. First, the metabolite should have a specific function, and its concentration should change in response to a specific stimulus. Second, the metabolite should be able to diffuse through the cell membrane and enter the cytoplasm. Third, the metabolite should be able to bind to a specific receptor or enzyme and modulate its activity. Fourth, the metabolite should be able to elicit a specific biological response when it is present at a certain concentration.

### Table 3. Lipidomic analysis of HeLa/MT and the indicated KO cells

| Parameters     | Without Mevalonate | With Mevalonate (3 mM) |
|----------------|--------------------|-------------------------|
|                | WT                 | CYP51A1 KO              | CYP51A1-DHCR24 DKO | MSMO1 KO | MSMO1-DHCR24 DKO |
| Squalene       | 0.0042             | 0.032                   | 0.0075             | 0.022    | 0.016             | 0.012    | 0.0051             | 0.028             | 0.006             | 0.022             |
| Lanosterol     | 0.08               | 5.36                    | 2.04               | 15.09    | 8.19              | 36.22    | 0.11               | 4.73              | 0.13              | 32.48             |
| 24,25-DHL      | 0.16               | 4.90                    | 6.73               | 21.45    | 0.15              | 0.01     | 0.15               | 4.69              | 0.03              | 0.04              |
| FF-MAS         | 0.05               | 3.04                    | 0.01               | 0.01     | 0.02              | 0.02     | 0.17               | 4.31              | 0.21              | 10.89             |
| T-MAS          | 0.19               | 16.03                   | 0.09               | 0.45     | 0.97              | 1.58     | 7.60               | 37.02             | 48.93             | 83.45             |
| Dihydro-T-MAS  | 0.23               | 15.03                   | 0.13               | 0.24     | 0.07              | 0.03     | 13.99              | 23.12             | 0.42              | 0.38              |
| Zymosterol     | 0.09               | 0.84                    | 0.02               | 0.02     | 0.01              | 0.01     | 0.03               | 0.03              | 0.02              | 0.02              |
| Dehydrolathosterol | 3.80         | 22.48                   | 0.94               | 0.88     | 3.15              | 2.52     | 1.27               | 1.22              | 2.54              | 8.13              |
| Lathosterol    | 1.18               | 4.78                    | 0.86               | 0.72     | 0.23              | 0.15     | 0.81               | 0.90              | 0.35              | 0.36              |
| 7-Dehydrocholesterol | 5.95       | 10.82                   | 3.29               | 2.65     | 2.96              | 1.92     | 4.19               | 4.84              | 1.38              | 14.00             |
| Desmosterol    | 0.25               | 1.38                    | 0.10               | 0.09     | 0.12              | 0.08     | 0.12               | 0.12              | 0.12              | 0.16              |
| Cholesterol    | 937                | 1056                    | 850                | 730      | 622               | 386      | 839                | 847               | 730               | 876               |

WT, CYP51A1 KO, CYP51A1-DHCR24 DKO, MSMO1 KO, and MSMO1-DHCR24 DKO HeLa/MT cells were depleted of sterol in medium A for 16 h, and then switched to medium A supplemented with or without 3 mM mevalonate for 5 h. The cells were collected and subjected to lipidomics analysis. The level of various sterol intermediates are shown as absolute level normalized with micromoles of PC.

Fig. 6. C4-dimethylated sterol intermediates show dramatic activity in feedback regulation. A: WT, MSMO1 KO, and MSMO1-DHCR24 DKO HeLa/MT cells were depleted of sterol by incubating in medium A for 16 h, and then switched to medium A supplemented with or without 3 mM of mevalonate for 5 h. The cells were collected and subjected to lipidomics analysis. The levels of each sterol intermediate were normalized to those in WT cells without mevalonate treatment. The absolute levels of various sterol intermediates are shown in Table 3. B: The cells were depleted of sterol by incubating in medium A for 16 h, and then switched to medium A supplemented with the indicated concentrations of mevalonate for 5 h. The cells were lysed, and Western blotting was carried out with monoclonal anti-HMGCR (IgG-A9), anti-DHCR24, and anti-GAPDH. C: Quantification of HMGCR in B. The HMGCR protein level was quantified by ImageJ and normalized to GAPDH. The HMGCR levels in WT, MSMO1 KO, or MSMO1-DHCR24 DKO HeLa/MT cells without treatment were defined as 1. D: The cells were depleted of sterol by incubating in medium B for 16 h, and then switched to medium A supplemented with the indicated concentrations of mevalonate for 4 h. The cells were lysed, and Western blotting was carried out with monoclonal anti-SREBP-2 (IgG-1D2), pSREBP-2, precursor of SREBP-2; nSREBP-2, nuclear form of SREBP-2. E: Quantification of SREBP-2 in D. The nuclear and precursor forms of SREBP-2 were quantified by ImageJ and the ratio of nSREBP-2 to total SREBP-2 [n/(n + p)] was calculated. The ratios in lanes 1, 5, and 9 were defined as 1, respectively.
potent activity toward a certain signaling event at the endogenous level. Second, its concentration should be subjected to quick and dramatic change at varying conditions. Lanosterol is certainly a signal molecule, as it promotes HMGR ubiquitination and degradation and does not inhibit SREBP-2 processing (18) (Fig. 5; supplemental Figs. S2, S3). In Table 1, we show that the lanosterol concentrations were increased by 27.6- and 61.5-fold in HeLa and HeLa/MT cells when treated with mevalonate, in sharp contrast to 7-dehydrocholesterol that was only increased by 2.8- and 2.24-fold, respectively.

It is shown that cholesterol regulates SREBP-2 processing through binding to SCAP and inducing SCAP conformational change (16). Because the cholesterol concentration is at least 1000-fold higher than other sterol intermediates, the mevalonate treatment did not dramatically increase total cellular cholesterol level. However, how ER cholesterol level is altered by mevalonate treatment remains unknown. In cells lacking DHCR24 (Fig. 4), CYP51A1 (Fig. 5), MSMO1, and both MSMO1 and DHCR24 (Fig. 6), SREBP-2 cleavage could still be blocked by mevalonate. These results demonstrate that, besides cholesterol, other sterols in the mevalonate pathway can suppress SREBP-2 processing. The C4-dimethylated sterols (not lanosterol) seem to inhibit SREBP-2 cleavage because they accumulate in MSMO1 KO cells where SREBP-2 cleavage is blocked. Table 1 also shows that these C4-dimethylated sterols, including 24,25-DHL, FF-MAS, T-MAS, and dihydro-T-MAS, were dramatically increased under mevalonate treatment. It is possible that multiple members among 24,25-DHL, FF-MAS, T-MAS, and dihydro-T-MAS can promote HMGR degradation and inhibit SREBP-2 cleavage.

Interestingly, the mammalian cells use no canonical Bloch or Kandutsch-Russell pathways, but use the cell type-specific hybrid Bloch and modified Kandutsch-Russell pathways instead (46). These C4-dimethylated sterols, despite different in kinds and amounts, can all feedback control cholesterol biosynthesis. However, our results could not rule out other sterol intermediates between zymosterol and cholesterol that may regulate HMGR or SREBP-2.

A previous study by Lange et al. (29) showed that exogenously added lanosterol did not, but 24,25-DHL did, decrease endogenous HMGR activity. In their experiments, they treated human foreskin fibroblasts for 1 h and measured HMGR activity. We treated HeLa/MT cells for 5 h and then directly examined the HMGR protein level by Western blotting. These experimental differences may lead to different results. The endogenous HMGR activity is regulated at the multiple levels including transcription, translation, degradation, and phosphorylation (9). The HMGR activity may not always reflect protein level. In addition, the exogenously added lanosterol needs to be transported to the ER to regulate HMGR, whereas the mevalonate-derived lanosterol is synthesized right in the ER. The spatial distribution of lanosterol might cause different conclusions as well.

It is unknown how lanosterol and other C4-dimethylated sterols regulate HMGR degradation and SREBP-2 cleavage. Why lanosterol specifically regulates HMGR but not the SCAP-SREBP-2 complex is another question to be addressed. It would be interesting to test whether lanosterol selectively binds HMGR instead of SCAP. Other C4-dimethylated sterols might bind both HMGR and SCAP. It is also possible that the C4-dimethylated sterols, except for lanosterol, bind Insig and regulate both pathways as 25-HC does. Identification of the structures of SCAP, HMGR, and Insig and their complexes with sterol ligands would be the key solution for these questions.

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REFERENCES

1. Porter, J. A., K. E. Young, and P. A. Beachy. 1996. Cholesterol modification of hedgehog signaling proteins in animal development. Science. 274: 253–259.
2. Xiao, X., J. A. F. Tang, C. Peng, Y. Wang, L. Fu, Z. P. Qiu, Y. Xiong, L. F. Yang, H. W. Cui, X. L. He, et al. 2017. Cholesterol modification of Smoothen is required for Hedgehog signaling. Mol. Cell. 66: 154–162.e110.
3. Luo, J., L. Y. Jiang, H. Yang, and B. L. Song. 2019. Intracellular cholesterol transport by sterol transfer proteins at membrane contact sites. Trends Biochem. Sci. 44: 273–292.
4. Barter, P., A. M. Gotto, J. C. LaRosa, J. Maroni, M. Szarek, S. M. Grundy, J. J. Kastelein, V. Bittner, and J. C. Fruchtart; Treating to New Targets Investigators. 2007. HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. N. Engl. J. Med. 357: 1301–1310.
5. Downs, J. R., M. Clearfield, S. Weis, E. Whitney, D. R. Shapiro, P. A. Beere, A. Langendorfer, E. A. Stein, W. Kruyer, and A. M. Gotto, Jr. 1998. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study, JAMA. 279: 1615–1622.
6. Zhang, Y. Y., Z. Y. Fu, J. Wei, W. Qi, G. Baintuola, J. Luo, Y. J. Meng, S. Y. Guo, H. Yiu, S. Y. Jiang, et al. 2018. A LIMA1 variant promotes low plasma LDL cholesterol and decreases intestinal cholesterol absorption. Science. 360: 1087–1092.
7. Cohen, J. C., E. Boerwinkle, T. H. Mosley, Jr., and H. H. Hobbs. 2006. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N. Engl. J. Med. 354: 1260–1272.
8. Goldstein, J. L., and M. S. Brown. 1990. Regulation of the mevalonate pathway. Nature. 343: 425–430.
9. Brown, M. S., and J. L. Goldstein. 1980. Multivalent feedback regulation of HMG Coa reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J. Lipid Res. 21: 505–517.
10. DeBoise-Boyd, R. A., and J. Ye. 2018. SREBP’s in lipid metabolism, insulin signaling, and beyond. Trends Biochem. Sci. 43: 358–368.
11. Song, B. L., N. Sever, and R. A. DeBoise-Boyd. 2005. Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG Coa reductase. Mol. Cell. 19: 829–840.
12. Cao, J., J. Wang, W. Qi, H. H. Miao, J. Wang, L. Ge, R. A. DeBoise-Boyd, J. J. Tang, B. L. Li, and B. L. Song. 2007. Uf1 is a cofactor of gp78 and plays a key role in cholesterol metabolism by regulating the stability of HMG-CoA reductase. Cell Metab. 6: 115–128.
13. Sever, N., B. L. Song, D. Yabe, J. L. Goldstein, M. S. Brown, and R. A. DeBoise-Boyd. 2003. Insig-dependent ubiquitination and degradation of mammalian 3-hydroxy-3-methylglutaryl-CoA reductase stimulated by sterols and geranylgeraniol. J. Biol. Chem. 278: 32470–32490.
14. Jiang, L. Y., W. Jiang, N. Tian, Y. N. Xiong, J. Liu, J. Wei, K. Y. Wu, J. Luo, X. J. Shi, and B. L. Song. 2018. Ring finger protein 145 (RNF145) is a ubiquitin ligase for sterol-induced degradation of HMG-CoA reductase. J. Biol. Chem. 293: 4047–4055.
15. Jo, Y., P. C. Lee, P. V. Sguigna, and R. A. DeBoise-Boyd. 2011. Sterol-induced degradation of HMG CoA reductase depends on interplay of two Insigs and two ubiquitin ligases, gp78 and TreC. Proc. Natl. Acad. Sci. USA. 108: 20503–20508.
16. Brown, M. S., A. Radhakrishnan, and J. L. Goldstein. 2018. Retrospective on cholesterol homeostasis: the central role of Scap. Annu. Rev. Biochem. 87: 783–807.
17. Gill, S., J. Stevenson, I. Kristiana, and A. J. Brown. 2011. Cholesterol-dependent degradation of squalene monooxygenase, a control point in cholesterol synthesis beyond HMG-CoA reductase. Cell Metab. 13: 260–273.
18. Song, B. L., N. B. Javitt, and R. A. DeBose-Boyd. 2005. Insig-mediated degradation of HMG CoA reductase stimulated by lanosterol, an intermediate in the synthesis of cholesterol. Cell Metab. 1: 179–189.
19. Gao, Y., Y. Zhou, J. L. Goldstein, M. S. Brown, and A. Radhakrishnan. 2018. Accumulation of 8,9-unsaturated sterols drives oligodendrocyte formation and remyelination. Nature. 575: 607–611.
20. Zhao, L., X. J. Chen, J. Zhu, Y. B. Xi, X. Yang, L. D. Hu, H. Ouyang, S. H. Patel, X. Jin, D. Lin, et al. 2015. Lanosterol reverses protein aggregation in cataracts. Nat. Struct. Mol. Biol. 22: 315–320.
21. Hubler, Z., D. Allimuthu, I. Bederman, M. S. Elitt, M. Madhavan, A. J. Brown, L. Sun, J. D. Feramisco, M. S. Brown, and J. L. Goldstein. 2018. Discovery of a potent HMG-CoA reductase degrader that eliminates statin-induced reductase accumulation and lowers cholesterol. Mol. Cell. 70: 1070–1083.
22. Liao, J., Y. Guan, C. Shi, D. Yao, F. Wang, S. M. Lam, G. Shui, and X. Cao. 2019. ACBD3 is required for FAPP2 transferring glucosylceramide through maintaining the Golgi integrity. J. Mol. Cell Biol. 11: 107–117.
23. Honda, A., K. Yamashita, H. Miyazaki, M. Shirai, T. Ikegami, G. Xu, M. Numazawa, T. Hara, and Y. Matsuoka. 2008. Highly sensitive analysis of sterol profiles in human serum by LC-ESI-MS/MS. J. Lipid Res. 49: 2063–2073.
24. Garcia, C. K., J. L. Goldstein, R. K. Pathak, R. G. Anderson, and M. S. Brown. 1994. Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle. Cell. 76: 865–873.
25. Maurer-Stroh, S., S. Wasniedl, and F. Eisenhaber. 2003. Protein prenyltransferases. Genome Biol. 4: 212.
26. Bykov, A. G., C. Y. Andersen, and L. Leonardsen. 2002. Role of meiosis activating sterols, MAS, in induced oocyte maturation. Mol. Cell. Endocrinol. 187: 189–196.
27. Keber, R., J. Acimovic, G. Majdic, H. Motaln, D. Rozman, and S. Horvat. 2013. Male germ cell-specific knockout of cholesterologenic cytochrome P450 lanosterol 14alpha-demethylase (Cyp51). J. Lipid Res. 54: 1653–1661.
28. Yang, C., J. G. McDonald, A. Patel, Y. Zhang, M. Umetani, F. Xu, E. J. Westover, D. F. Covey, D. J. Mangelsdorf, J. C. Cohen, et al. 2006. Sterol intermediates from cholesterol biosynthetic pathway as liver X receptor ligands. J. Biol. Chem. 281: 27816–27826.
29. Spann, N. J., L. X. Gamrke, J. G. McDonald, D. S. Myers, S. B. Milne, N. Shibata, D. Reichart, J. N. Fox, I. Shaked, D. Heudobler, et al. 2012. Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. Cell. 151: 138–152.
30. Faust, J., and M. Krieger. 1987. Expression of specific high capacity mevalonate transport in a Chinese hamster cell variant. J. Biol. Chem. 262: 1996–2004.
31. Brown, M. S., and J. L. Goldstein. 2012. Scientific side trips: six excurions from the beaten path. J. Biol. Chem. 287: 22418–22435.
32. Mitsche, M. A., J. G. McDonald, H. H. Hobbs, and J. C. Cohen. 2015. Flux analysis of cholesterol biosynthesis in vivo reveals multiple tissue- and cell-type specific pathways. eLife. 4: e07999.