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# Increased bile lithogenicity by SCP2 via HMGCR and CYP7A1 regulation in human hepatocytes

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***Background/aims:*** *Lithogenic bile is a major cause of cholesterol gallstones, and hypersecretion of biliary cholesterol is believed to be an important cause of lithogenic bile. Sterol carrier protein 2 plays a key role in cholesterol trafficking and may regulate lipid metabolism in hepatocytes.* ***Materials and Methods:*** *Human sterol carrier protein 2 cDNA was cloned and shRNAs against sterol carrier protein 2 were synthesized. Sterol carrier protein 2-modified hepatocyte models were then set up using adenoviral vectors to study its regulatory function in lipid metabolism. Following overexpression and knockdown of sterol carrier protein 2, HMGCR and CYP7A1 expression as well as the secretion of bile lipids were studied in hepatocytes.* ***Results:*** *The expression of HMGCR was enhanced by sterol carrier protein 2 overexpression and downregulated by sterol carrier protein 2 knockdown. The concentration of cholesterol in the supernatant was elevated under sterol carrier protein 2 overexpression and decreased under sterol carrier protein 2 downregulation. Although sterol carrier protein 2 overexpression could repress the expression of CYP7A1, no changes were noted in total bile acid concentration. Thus, the expression of sterol carrier protein 2 could influence bile lithogenicity in the sterol carrier protein 2-modified hepatocyte models.* ***Conclusions:*** *Sterol carrier protein 2 may function as a moderator of HMGCR in human hepatocytes. Likely influences cholesterol metabolism and bile lithogenicity of human liver cells by regulating the expression of HMGCR and CYP7A1.*

**Key words:** Human hepatocyte, sterol carrier protein 2, bile lithogenicity, lipid metabolism

## SCP2, insan hepatositlerinde HMGCR ve CYP7A1'i regüle ederek safra litojenisitesini arttırır

***Giriş ve Amaç:*** *Litojenik safra kolesterol taşlarının en önemli nedenidir ve safrada kolesterolün aşırı salgılanmasının litojenik safranın önemli bir nedeni olduğuna inanılmaktadır. Sterol taşıyıcı protein 2 kolestorol taşınmasında anahtar role sahiptir ve hepatositlerde lipid metaboizmasını regüle eder.* ***Gereç ve Yöntem:*** *İnsan sterol taşıyıcı protein 2 cDNA klonlanmış ve buna karşı shRNAs sentezlenmiştir, ertesinde adenovirüs vektörler ile oluşturulan sterol taşıyıcı protein 2-modifiye hepatosit modelleri kullanılarak regülatuvar fonksiyonları araştırılmıştır. Sterol taşıyıcı protein 2'nin aşırı ekspresyonunda ve silinmesinde, HMGCR ve CYP7A1 ekspresyonları ve hepatositlerden safra lipidlerinin sentezi incelenmiştir.* ***Bulgular:*** *HMGCR ekspresyonu, sterol taşıyıcı protein 2 aşırı ekspresyonu ile artmış, silinmesi ile azaldığı tespit edilmiştir. Ayrıca sterol taşıyıcı protein 2 aşırı ekspresyonunda supernatan içindeki kolesterol konsantrasyonun arttığı, baskılanması halinde azaldığı bulundu. Sterol taşıyıcı protein 2 aşırı ekspresyonu CYP7A1 ekspresyonunu baskılamasına rağmen total safra asidi konsantrasyonunda değişiklik tespit edilmedi. Sterol taşıyıcı protein 2'nin, modifiye hepatosit modellerinde safra litojenisitesini etkileyebildiği bulundu.* ***Sonuç:*** *Sterol taşıyıcı protein 2 hepatositlerde HMGCR'ın modülatörü olabilir. Sterol taşıyıcı protein 2 kolesterol metabolizmasını etkileyebilir ve hepatositlerdeki HMGCR ve CYP7A1 ekspresyonunu etkileyerek safranın litojenistesini değiştirebilir.*

**Anahtar kelimeler:** İnsan hepatositi, sterol taşıyıcı protein 2, safra litojenisitesi, lipid metaboizması

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## INTRODUCTION

Gallstone disease is an extremely common gastrointestinal condition. It is usually multifactorial in origin, and the etiology of this disease is related to a complex interaction of environmental and genetic factors (1–3). The worldwide prevalence rates range between 5% and 20%, but maybe as high as 70% in American Indian females.

Over 90% of gallstones consist mainly of cholesterol and are formed within the gallbladder. Cholesterol hypersaturation of bile is a prerequisite for the formation of such stones. Bile mainly consists of water and three lipid species: cholesterol, phospholipids and bile salts. Hypersecretion of cholesterol in bile leading to the formation of lithogenic bile is believed to be the major cause of cholesterol gallstones (4). Sterol carrier protein 2 (SCP2), also called nonspecific lipid transfer protein, is a 13.2 KD base protein and exists in peroxisomes, mitochondria, the endoplasmic reticulum and in the cytoplasm (5-7). In its role as a transporter, this protein participates in the transportation of cholesterol inside the cell and through the cytoplasm membrane, (8,9) as well as in the rapid transportation of newly synthesized cholesterol from the endoplasmic reticulum into bile without the intervention of the cytomicrotubule system and Golgi bodies (10). Additionally, some researchers indicate that it may have a role in the biosynthesis of cholesterol, (11-13) and in the transformation of cholesterol to bile acids (14, 15), cholesterol esters (16) and sterols (17). Our former findings indicated that SCP2 was overexpressed in patients with hereditary cholesterol gallstones when compared to patients with non-hereditary cholesterol gallstones, and that SCP2 may be a potential genetic factor that contributes to the formation of cholesterol gallstones (18). Hence, the hypersecretion of biliary cholesterol and formation of lithogenic bile may explain the mechanism of cholesterol stone formation in the gallbladder.

It is widely accepted that SCP2 is a transporter for lipids, but we suspected that SCP2 may play a role in the regulation of cholesterol metabolism. Therefore the expression of critical genes related to lipid metabolism and bile lipid secretion in hepatocytes was studied, under SCP overexpression and knockdown to evaluate the role of SCP2 in the regulation of lipid metabolism.

## MATERIALS and METHODS

### Human SCP2 cDNA cloning and sequencing

A PCR-based cloning strategy was used to isolate a cDNA fragment encoding SCP2 protein. According to the human SCP2 cDNA sequence [gi:432978] from Genebank, primers were designed using Oligo 6.0 software, and endonuclease restriction sequences (BamHI and EcoRI, Takara) were added at the 5' end of upper primer and lower primer respectively. The upper primer is 5'AAAGAATTCAGAGGCAAGTTC CTGGTG, and the lower primer is 5'-AAAGGATCCTTCTTCAGAGCTTAG CGTTG. The results of DNA sequencing depicted that the SCP2 cDNA sequence had the same sequence as that [gi: 432978] in the Genebank. Finally, the correct Human SCP2 cDNA clone was obtained from thepSCP2-IRES2-EGFP Vector.

### Construction of the SCP2 shRNA with two-step PCR method

A two-step PCR method was used to construct SCP2 shRNA. With the use of online software (http://www.promega.com/siRNADesigner/default.htm), shRNA primers were designed according to the human SCP2 cDNA sequence [gi: 432978] from Genebank. The primers are shown in Table1. The template of the first-step PCR was pGEMZeo-U6 with the human U6 promoter. The template of the second step PCR was the products of the first PCR reaction,which were diluted by 100-fold. Four SCP2 shRNAs were obtained; these were pMD18T-SCP2sh1, pMD18T-SCP2sh2, pMD18T -SCP2sh3 and pMD18T-SCP2ssh.

### Cell lines and culture

293T cells were grown in DMEM (Irvine Scientific, Santa Ana, California) supplemented with 10% fetal calf serum (Irvine Scientific) and 100 U/mL of penicillin/streptomycin. Chang liver cells were cultivated in RPMI1640 (Irvine Scientific, Santa Ana, California) with 10% fetal calf serum (Irvine Scientific) and 100 U/mL of penicillin/streptomycin.

### Evaluation of inhibitory effects of SCP2 shRNAs on exogenous and endogenous expression of SCP2

Lipofectamine2000 (Invitrogen) was used for cell line transfection. 293T cells were co-transfected with pSCP2-IRES2-EGFP and pMD18T-SCP2shRNAs to detect any inhibitory effect on the exogenous expression of SCP2. Chang liver cells were only transfected with pMD18T-SCP2shRNAs to detect an inhibitory effect on the endogenous expression of SCP2. The mRNA expression of SCP2 was detected by real-time quantitative RT-PCR, and the primers

are seen in the Table 2. A proper shRNA was chosen from the above three SCP2shRNAs, which had the best knockdown effect on both the endogenous and exogenous expression of SCP2.

### Construction of replication defective adenoviral vectors carrying the SCP2 cDNA and the short hairpin sequence of SCP2 including human U6 promoter

A PCR-based cloning strategy was used to isolate the SCP2-IRES2-EGFP sequence fragment from pSCP2-IRES2-EGFP. Primers were designed using Oligo 6.0 software and endonuclease restriction sequences (EcoRI and SalI, Takara) were added at the 5' end of the upper and lower primers respectively. The upper primer used was 5'-AAAGAATTCAGAGGCAAGTT CCTGGTG and the lower primer used was 5'-CCCGTC GACGGCCGCTTTACTTGTACAGC. The sequence of SCP2-IRES2-EGFP was subcloned into pDC316. The SCP2-shRNA sequence was then cut with endonucleases (Hind III and BamH I) from pMD18T-SCP2 shRNA and subcloned into pDC312. The Admax Adenoviral Vector System was used to generate replication-defective adenoviral vectors. We successfully constructed replication-defective adenoviral vectors AdSCP2-IRES2-EGFP, Adnull, AdshRNASCP2 and AdsshRNASCP2, which carried the cDNA of SCP2 gene and SCP2-shRNAs. The AdSCP2-IRES2-EGFP, Adnull, AdshRNASCP2 and AdsshRNA SCP2 titers were $3x10^8$ pfu/ml, $2x10^8$ pfu/ml, $1.5x10^8$ pfu/ml, $3.1x10^8$ pfu/l, respectively.

### Isolation and culture of primary human hepatocytes

Normal human liver tissue was obtained from 5 different patients with liver cancer undergoing liver resection. Approval to use portions of resected human liver specimens for research was obtained from the ethics committee at our hospital. Where applicable, patients gave informed consent. Hepatocytes were isolated by the digestive and mechanical methods. Cells were then cultured in a humidified 5% $CO_2$ incubator at 37°C. After 24 h, the medium was changed to 5% FBS medium. At 72 h the medium was replaced with SFM. Thereafter, the medium was replaced at least every 48 h with freshly prepared SFM.

### AdSCP2-IRES2-EGFP, Adnull, AdshRNASCP2 and AdsshRNASCP2 were transfected into primary cultured hepatocyte

After the hepatocytes were digested and transferred to culture plates, DMEM medium was added to the plates. After 80% fusion, hepatocytes were

**Table 1.** The list of used primers in two-step PCR for shRNA construction

| shRNAs | Sequences | Length |
|---|---|---|
| SCP2shRNA1 | Step1 primer: 5'aga gaa ctt/aag ttt ctt ctc aat ctc c/gg tgt ttc gtc ctt tcc a<br>Step2 primer: 5'gga att caa aaa a/gg aga ttg aga aga aac tt/a gag aac tt | 460bp |
| SCP2shRNA2 | Step1 primer: 5'aga gaa ctt/att ctt cac atc cac cac c/gg tgt ttc gtc ctt tcc a<br>Step2 primer: 5'gga att caa aaa a/gg tgg tgg atg tga aga at/a gag aac tt | 460bp |
| SCP2 shRNA3 | Step1 primer: 5'aga gaa ctt/taa ctt cat agc gag acc c/gg tgt ttc gtc ctt tcc a<br>Step2 primer: 5'gga att caa aaa a/gg gtc tcg cta tga agt ta/a gag aac tt | 460bp |
| SCP2 sshRNA | Step1 primer: 5'aga gaa ctt/act att cac caa cct cct c/gg tgt ttc gtc ctt tcc a<br>Step2 primer: 5'gga att caa aaa a/ga gga ggt tgg tga ata gt/a gag aac tt | 460bp |
| Universal upper primer | 5'gca gac act gct cgg tag tt | |

**Table 2.** The list of used primers for SCP2, HMGCR and CYP7A1 mRNA detection

| Gene | Sequences | Length |
|---|---|---|
| GAPDH | Upper: GGGGAGCCAAAAGGGTCATCATCT<br>Lower:GACGCCTGCTTCACCACCTTCTTG | 457bp |
| SCP2 | Upper: AGCTCTGCAAGTGATGGATTTAAG<br>Lower: CGACTGAGGATTCATTTTACCAG | 276bp |
| HMGCR | Upper: TACCATGTCAGGGGTACGTC<br>Lower:CAAGCCTAGAGACATAATCATC | 247 bp |
| CYP7A1 | Upper: ATGGGTTTTCCGGAAGCCGCCAGTT | 432 bp |

used for transfection. The optimal transfection concentration of AdSCP2-IRES2-EGFP was 100 MOI. Cells were collected 24 hours after transfection to extract total RNA and 48 hours after transfection to extract total protein. The supernatants were then collected for high pressure liquid chromatography (HPLC) analysis.

### Analysis of SCP2, HMGCR and CYP7A1 mRNA using real-time quantitative RT-PCR

Total RNA was isolated using the TRIzol reagent. The mRNA expression of SCP2, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) and 7alpha-hydroxylase (CYP7A1) was detected by using real-time RT-PCR, and the primers are seen in Table 2. PCR was performed as follows: step 1 94°C, 2 min; step 2 94°C, 30 sec; step 3 gradient from 54°C to 56°C, 1 min; step 4 72°C, 45 sec; step 5 80°C, 1 sec; step 6 Plate read; step 7 Repeat step 2 39 more times; step 8 72°C, 3 min; step 9 Melting curve analysis: 70°C-95°C, 0.5°C/read, 1 sec hold; step 10 72°C,3 min; step 11 20°C, Hold which then concluded the process. The PCR products were 276 bp, 247 bp and 423 bp.

### The detection of SCP2 protein level with Western Blot

Tissues were lased with a lysis buffer (50 mM Tris PH7.4,150 mM NaCl,0.5% Triton X-100,1 mM EDTA,1 mM PMSF). The total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred onto a polyvinylidene difuoridemembrane (Millipore, Bedford, MA, USA). After blocking non-specific binding sites with 5% non-fat milk, the membrane was incubated with primary antibodies for 1 h at room temperature. The primary antibodies used in this experiment were as follows: anti-SCP2 antibody (Donated by Dr Schroeder, Texas A&M University, 1:1000 dilution) and anti-GAPDH antibody (Santa Cruz Biotechnology, 1:2000 dilution). After washing, blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG, and immunoreactive bands were visualized with the Western Blotting Luminol Reagent kit (Santa Cruz Biotechnology Inc).

### Analysis of bile acids, biliary cholesterol, phospholipids and lithogenic index (LI) in cell culture samples using high performance liquid chromatography

Frozen samples were quickly thawed, centrifuged for 5 min at 1000 rpm in a centrifuge, and filtered through a 0.45-um filter. HPLC was carried out using a Kromasil C18 (250mmx4.6mm 10μm) column. The column was equilibrated with 0.05mol/L sodium dihydrogen phosphate-methanol (25:75) buffer, pH 3.0. 100 ul of culture media was injected into the machine. The flow rate was maintained at 1.0 mL/min. We detected signals with the UV detector at wavelength of 210 nm. Cholesterol, lecithin, glycocholate acid (GCA), taurochenodeoxycholate acid (TCDCA), taurocholate acid (TCA), cholate acid (CA) and chenodeoxycholate acid (CDCA) (Sigma) were used as measurement standards for HPLC. The lithogenic index was then calculated using the Thomas and Hofmann method (19).

### Data analysis

All data are expressed as the mean±standard deviation. Differencesbetween control and experimental groups were evaluated by one-way analysis of variance (ANOVA). $p<0.05$ was considered statistically significant ($a = 0.05$).

## RESULTS

### pMD18T-SCP2sh1 and pMD18T-SCP2sh3 can inhibit the exogenous expression of SCP2 in 293T cells transfected with pSCP2-IRES2-EGFP, and the endogenous expression of SCP2 in Chang liver cells

Real-time RT-PCR was performed to detect exogenous expression of SCP2 in 293T cells transfected with pSCP2-IRES2-EGFP. Compared with the random shRNA control group, small hairpin RNAs (SCP2sh1, SCP2sh2, and SCP2sh3) significantly reduced the expression of exogenous SCP2 ($p<0.05$). The inhibitory rates were 71%, 75% and 62% respectively, shown in Figure 1 C and in Table 3.

In order to confirm endogenous inhibitory effects, pMD18T-SCP2sh1, pMD18T-SCP2sh2, pMD18T-SCP2sh3 or pMD18T-SCP2ssh were used to transfect Chang liver cells. Compared with the random shRNA control group, SCP2sh1and SCP2sh3 significantly reduced the expression of SCP2 ($p <0.05$) and inhibitory rates were 76% and 64% respectively, but the inhibitory rate of SCP2sh2 was only 9%, shown in Figure 1C and Table 3.

### SCP2 overexpression and knockdown cellular models were successfully set up in primary hepatocytes

We used real-time RT-PCR to detect the expression of SCP2 mRNA in hepatocytes 24 hours after

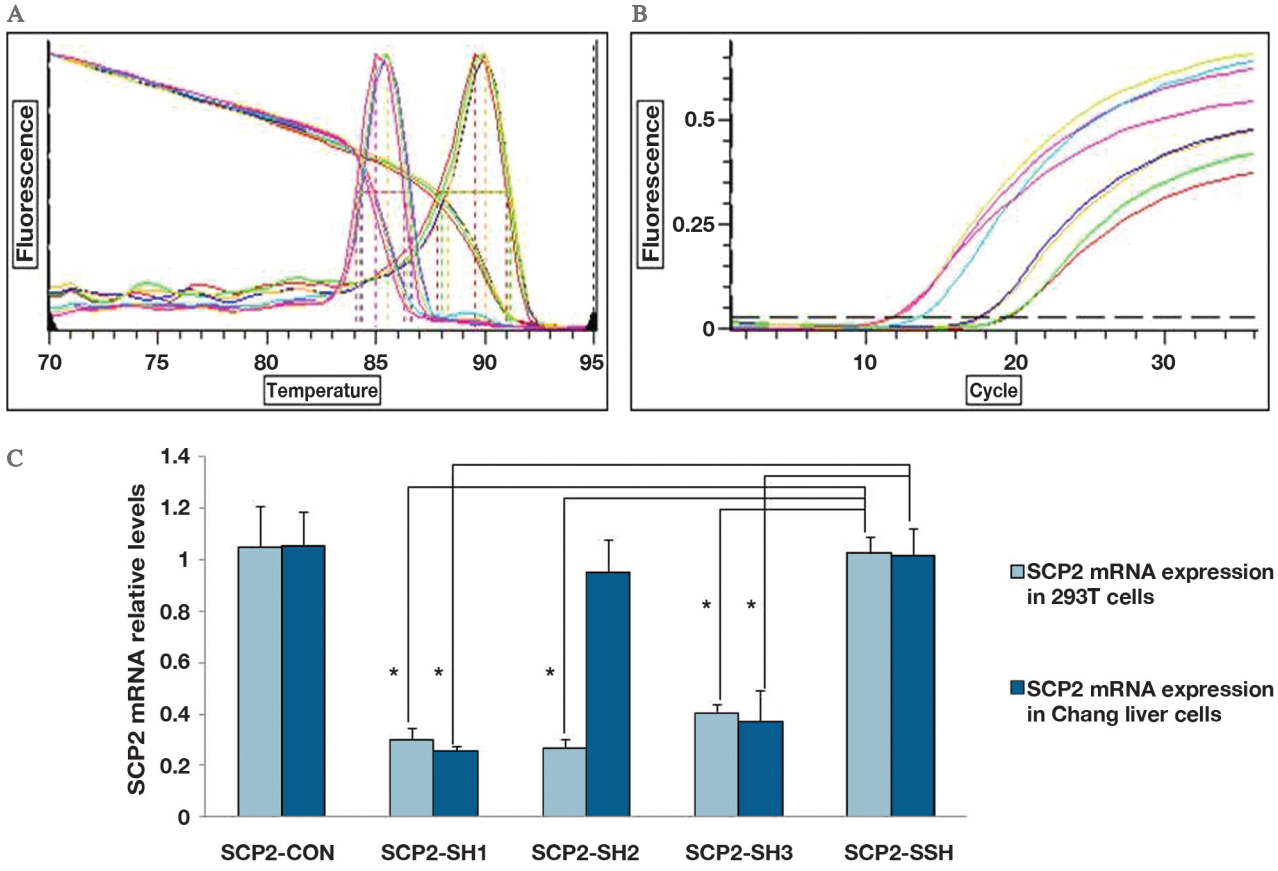


**Figure 1.** Melting curves and fluorescence amplification curves of SCP2 and GAPDH are shown in A and B. Small hairpin RNAs (SCP2sh1 and SCP2sh3) significantly reduced the expression of SCP2 in both, 293T cells transfected with pSCP2-IRES2-EGFP and Chang liver cells, but SCP2sh2 could only suppress the exogenous expression of SCP2 in 293T cells.
* indicates p<0.05. The experiment was repeated independently three times.

**Table 3.** The expression levels of SCP2 mRNA in 293T and Chang liver cell lines

| Group | SCP2 mRNA expression in 293T [a] | SCP2 mRNA expression in Chang liver [b] |
|---|---|---|
| SCP2-CON | 1.0513±0.1298 | 1.0566±0.1565 |
| SCP2-SH1 | 0.3005±0.0188*** | 0.2570±0.0466** |
| SCP2-SH2 | 0.2679±0.1290** | 0.9529±0.0331 |
| SCP2-SH3 | 0.4057±0.1189* | 0.3718±0.0339* |
| SCP2-SSH | 1.0273±0.1035 | 1.0180±0.06289 |

[a] compared with SCP2-SSH,***,** and * p<0.05 [b] compared with SCP2-SSH,** and * p<0.05

transfection by AdSCP2-IRES2-EGFP and AdshRNASCP2. Compared with the Adnull group, SCP2 mRNA was overexpressed in the AdSCP2-IRES2-EGFP group (p<0.05). Compared with the AdsshRNASCP2 group, mRNA expression of SCP2 in the AdshRNASCP2 group was significantly lower (p<0.05) and the inhibitory rate was 84%. There were no significant differences in the expression of SCP2 mRNA between controls, Adnull and AdsshRNA groups (p>0.05), as shown in Table 4 and Figure 2 C.

The Western blot was used to detect SCP2 protein expression in hepatocytes 48 hours after liver cells were transfected by AdSCP2-IRES2-EGFP and AdshRNASCP2. Compared with the Adnull group, we found that SCP2 was overexpressed in the AdSCP2-IRES2-EGFP group (p<0.05). Compared with the AdsshRNASCP2 group, SCP2 expression

in the AdshRNASCP2 group dropped significantly ($p<0.05$). There were no significant differences in SCP2 expression between the control, Adnull and AdsshRNA group ($p>0.05$). The results of SCP2 protein were consistent with those of SCP2 mRNA, as shown in Table 4, and Figure 2 D and 2 E.

### The mRNA expression of HMGCR, CYP7A1 in primary hepatocyets transfected with AdSCP2-IRES2-EGFP and AdshRNASCP2

Real-time RT-PCR was used to detect the expression of HMGCR and CYP7A1 mRNA in primary hepatocytes 24 hours after hepatocytes were transfected by AdSCP2-IRES2-EGFP and AdshRNASCP2. Compared with the Adnull group, HMGCR mRNA was overexpressed in the AdSCP2-IRES2-EGFP group ($p<0.05$). Compared with the AdsshRNASCP2 group, HMGCR mRNA expression in the AdshRNASCP2 group was significantly lower ($p<0.05$). No significant differences in HMGCR mRNA expression were noted between the control, Adnull and AdsshRNA groups ($p>0.05$). Compared with the Adnull group, the expression of CYP7A1 mRNA in the AdSCP2-IRES2-EGFP group was higher ($p<0.05$). There were no significant differences in CYP7A1 mRNA expression between th AdshRNASCP2 and AdsshRNASCP2 group ($p>0.05$). No significant differences in CYP7A1 mRNA expression were noted between the control, Adnull and AdsshRNA groups ($p> 0.05$), as shown in Table 5 and Figure 3.

### Analysis of bile acids, cholesterol and phospholipids in cell culture medium by using HPLC

We separated and detected 7 components in the culture supernatant of hepatocytes in the above chromatographic conditions after primary hepatocytes were infected with AdSCP2-IRES2-EGFP, AdshRNASCP2 for 48 hours. The values of TCA, TCDCA, GCA, CA and CDCA were added together to represent the total bile acid (TBA) for further analysis. Compared with the Adnull group, we found that the concentration of cholesterol was higher in the AdSCP2-IRES2-EGFP group ($p<0.05$). Compared with the AdsshRNASCP2 group, the concentration of cholesterol in the AdshRNASCP2 group was significantly lower ($p<0.05$).There were no significant differences in the concentration of cholesterol between the control, Adnull and AdsshRNA group ($p>0.05$). There was no significant difference in the concentration of TBA between the AdSCP2-IRES2-EGFP and Adnull groups ($p>0.05$), and the AdshRNASCP2 and AdsshRNASCP2 groups ($p>0.05$). Compared with the Adnull group, we found that the concentration of lecithin was higher in the AdSCP2-IRES2-EGFP group ($p<0.05$). Compared with the AdsshRNASCP2 group, the concentration of lecithin in the AdshRNASCP2 group was significantly lower ($p<0.05$). There were no significant differences in the concentration of lecithin between the control, Adnull and AdsshRNA group ($p> 0.05$), as shown in Table 6 and Figure 4 A.

Compared with the Adnull group, we found that the LI was higher in the AdSCP2-IRES2-EGFP group ($p<0.05$). Compared with the AdsshRNASCP2 group, the LI in the AdshRNASCP2 group was significantly lower ($p<0.05$). There were no significant differences in the LI between the control, Adnull and AdsshRNA groups ($p>0.05$), as shown in Table 6 and Figure 4 B.

## DISCUSSION

Cholesterol gallstone formation is a complicated disease entity that involves a variety of factors. Abnormal metabolism of cholesterol and supersaturation of bile cholesterol are the major causes of stone formation. Barth CA et al. (20) concluded that hepatocytes in culture show a functional po-

**Table 4.** The expression levels of SCP2 mRNA and protein in primary hepatocytes

| Group | SCP2 protein [a] | SCP2mRNA [b] |
|---|---|---|
| Control | 0.6456±0.0820 | 1.0780±0.1351 |
| AdSCP2-IRES2-EGFP | 9.7191±0.4826** | 20.9410±6.5096** |
| Adnull | 0.6423±0.1050 | 1.0263±0.1285 |
| AdshRNASCP2 | 0.0830±0.1473* | 0.1430±0.0200* |
| AdsshRNASCP2 | 0.5190±0.1053 | 1.0780±0.1100 |

[a] **compared with Adnull group, $p<0.05$; *compared with AdsshRNASCP2 group, $p<0.05$

[b] **compared with Adnull group, $p<0.05$; *compared with AdsshRNASCP2 group, $p<0.05$

larity permitting the transcellular transport of substances bound for biliary secretion. Thus, liver cells may maintain some characteristics of the liver and can be considered as a platform to study lipid metabolism in vitro. In this study, SCP2 overexpression and knockdown was performed in hepatocyte models in order to study the regulatory function of SCP2 in lipid metabolism.

Ito et al. (21) conducted an analysis of three regulatory enzymes in cholesterol metabolism, and studied cholesterol levels and sterol carrier protein2/nonspecific lipid transfer protein (SCP2/nsLTP) levels by using liver biopsy samples from Japanese patients with cholesterol gallstones who had undergone cholecystectomies. Their results suggested that intracellular cholesterol transport was enhan-

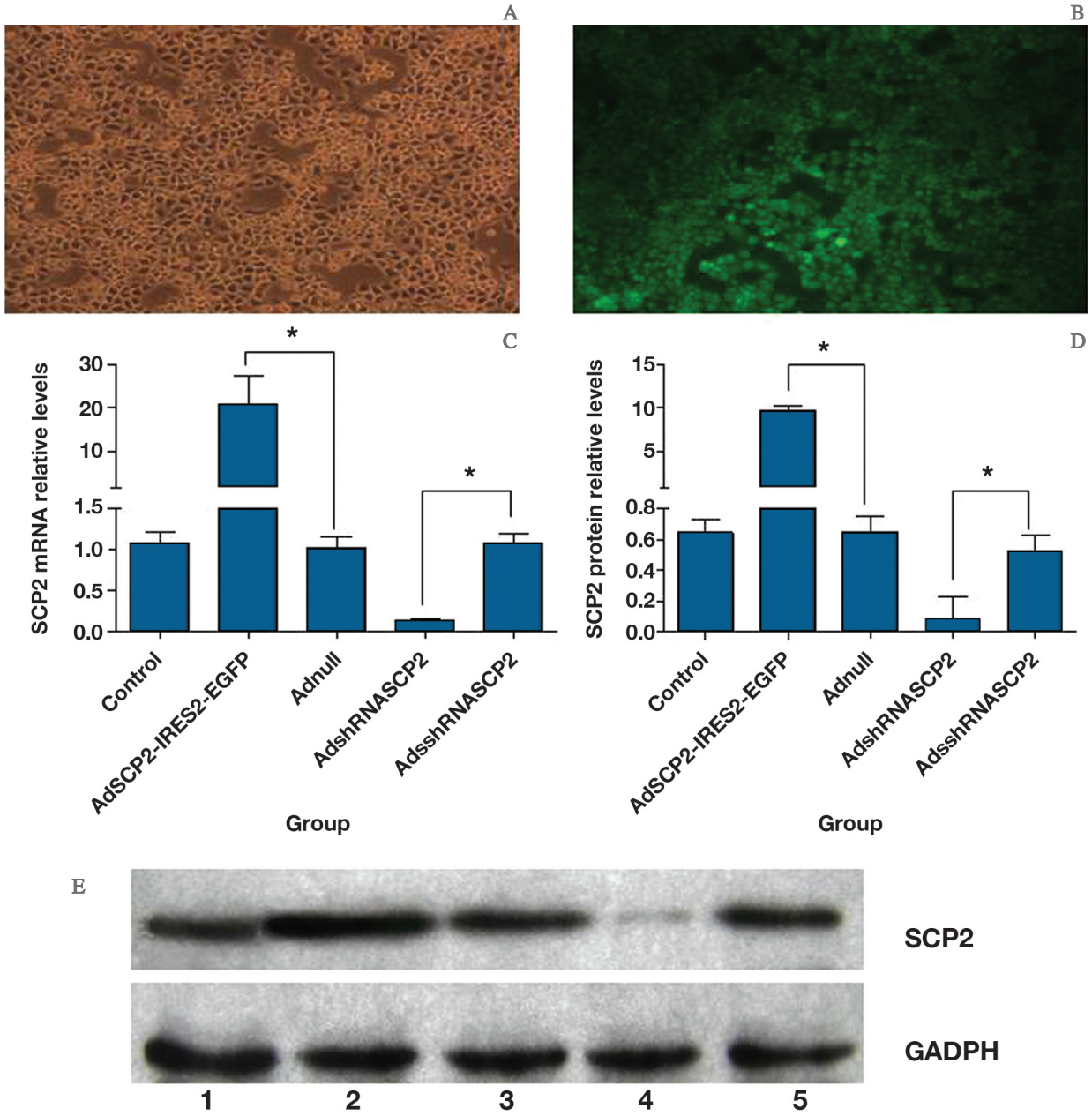


**Figure 2.** Green fluorescence was observed under a fluorescent microscope 48 h after primary hepatocytes were transfected with AdSCP2-IRES2-EGFP (panel A: phase contrast microscope, panel B: fluorescence microscope). SCP2 was overexpressed in the AdSCP2-IRES2-EGFP group, and the expression of SCP2 in the AdshRNASCP2 group decreased (C and D). A Western blot was used to detect SCP2 expression. Lanes 1, 2, 3, 4 and 5 represent Control, AdSCP2-IRES2-EGFP, Adnull, AdshRNASCP2 and AdsshRNASCP2 group, respectively (E).

* indicates $p<0.05$. The experiment was repeated independently three times.

**Table 5.** The relative expression levels of HMGCR and CYP7A1mRNA in primary hepatocytes

| Group | HMGCR [a] | CYP7A1 [b] |
|---|---|---|
| Control | 1.0405±0.0811 | 1.0569±0.1284 |
| AdSCP2-IRES2-EGFP | 1.4098±0.0378** | 0.8447±0.0457** |
| Adnull | 1.0402±0.1607 | 1.0254±0.0378 |
| AdshRNASCP2 | 0.8280±0.0365* | 1.1805±0.0765* |
| AdsshRNASCP2 | 0.9751±0.0328 | 1.0756±0.1133 |

[a] **compared with Adnull group, P<0.05;*compared with AdsshRNASCP2 group, P<0.05

[b] **compared with Adnull group, P<0.05;*compared with AdsshRNASCP2 group, P>0.05

**Table 6.** Analysis of bile acids, cholesterol and phospholipids in culture supernatant of hepatocytes using HPLC and LI

| Group | CHO [a] (mmol/L) | TBA [b] (mmol/L) | PL [c] (mmol/L) | LI[d] |
|---|---|---|---|---|
| Control | 0.8451±0.0382 | 9.2532±0.3881 | 0.3635±0.0252 | 0.4728±0.0276 |
| AdSCP2-IRES2-EGFP | 1.6561±0.0970** | 9.0499±0.3155** | 0.4194±0.0103** | 0.8371±0.1161** |
| Adnull | 0.7891±0.0457 | 8.7993±0.5072 | 0.3575±0.0207 | 0.4318±0.0449 |
| AdshRNASCP2 | 0.4632±0.1562* | 8.9013±0.3041* | 0.3071±0.0141* | 0.2734±0.0629* |
| AdsshRNASCP2 | 0.8274±0.0692 | 9.2491±0.1439 | 0.3825±0.0287 | 0.4625±0.0210 |

CHO: Cholesterol. TBA: Total bile acid. PL: Phospholipids. HPLC: High pressure liquid chromatography.

[a] **compared with Adnull group, P<0.05; *compared with AdsshRNASCP2 group, P<0.05

[b] **compared with Adnull group, P>0.05; *compared with AdsshRNASCP2 group, P>0.05

[c] **compared with Adnull group, P<0.05; *compared with AdsshRNASCP2 group, P<0.05

[d] **compared with Adnull group, P<0.05; *compared with AdsshRNASCP2 group, P<0.05

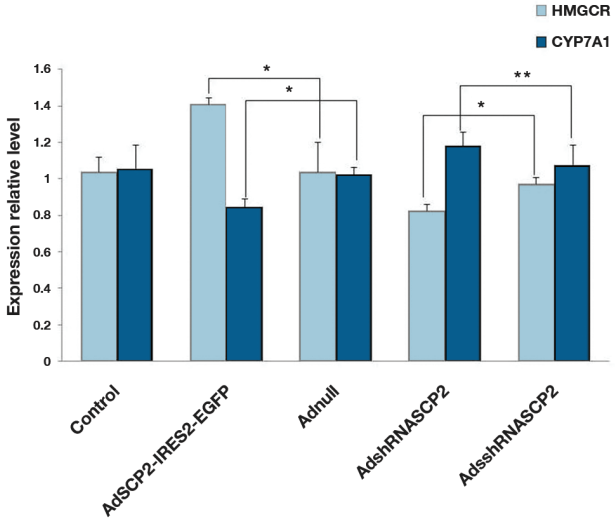


**Figure 3.** HMGCR mRNA was overexpressed in the AdSCP2-IRES2-EGFP group, but the mRNA expression of HMGCR in the AdshRNASCP2 group decreased significantly. The mRNA expression of CYP7A1 in the AdSCP2-IRES2-EGFP group increased.

* indicates p<0.05 and ** represents p>0.05. The experiment was repeated independently three times.

ced in patients with cholesterol gallstones. Animal experiments confirm that SCP2 plays an essential role in the transportation of newly synthesized cholesterol into bile, and that SCP2 can rapidly transfer cholesterol from the endoplasmic reticulum directly into the bile without the involvement of the cellular microtubule system and the Golgi (22). Fuchs et al. (14, 23) observed the phenomenon in the stone-susceptible mice, and noted that the SCP2 protein and mRNA levels rose at the same time and concluded that the transcriptional upregulation of SCP2 led to the higher levels of SCP2 in hepatocytes, thus increasing bile cholesterol and promoting gallstone formation.

At present, SCP2 is generally considered a lipid transporter, but we think that SCP2 is likely to have a role in cholesterol metabolism, especially in the biosynthesis of cholesterol and the transformation from cholesterol esters to cholesterol, bile acid and steroid hormone. In order to further clarify the relationships between SCP2 and other critical genes related to cholesterol metabolism, we detected the expression of HMGCR and CYP7A1 in SCP2 overexpression and knockdown hepatocyte models. We noted that the expression of HMGCR was enhanced by SCP2 overexpression and downregulated by SCP2 knockdown. We also observed that the concentration of cholesterol in the super-

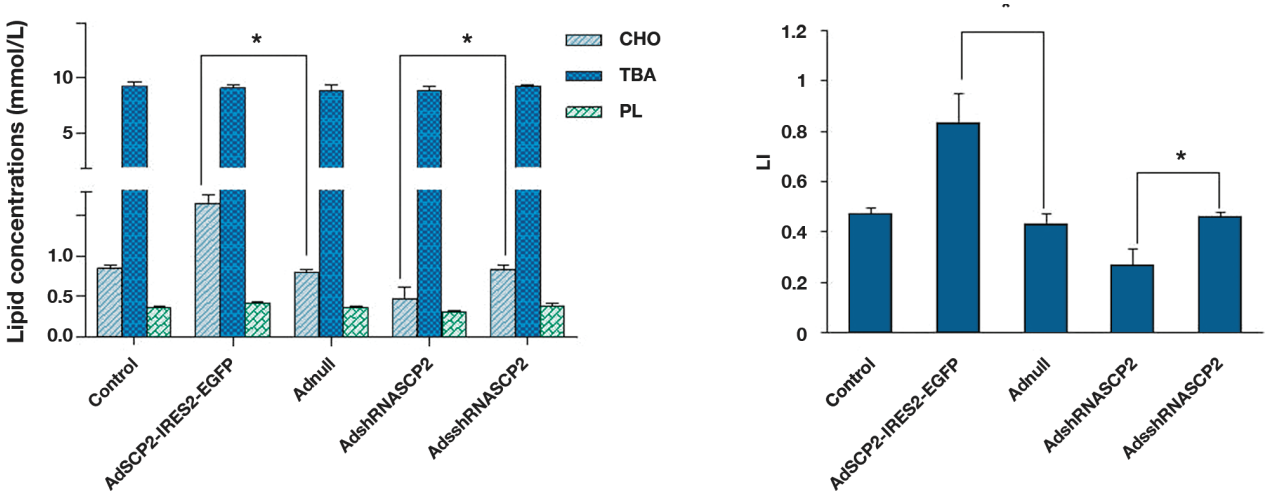


**Figure 4.** The concentration of cholesterol increased in the AdSCP2-IRES2-EGFP group, but that of cholesterol in the AdshRNASCP2 group decreased significantly **(A).** The concentration of lecithin increased in the AdSCP2-IRES2-EGFP group, and that of lecithin in the AdshRNASCP2 group decreased significantly **(A)**. LI went up in the AdSCP2-IRES2-EGFP group, but LI in the AdshRNASCP2 group decreased significantly **(B).**
* indicates $p<0.05$. The experiment was repeated independently three times.

natant was increased by SCP2 overexpression, and decreased by SCP2 downregulation. These findings indicate that SCP2 may function as a moderator for HMGCR in hepatocytes. Although SCP2 overexpression could repress the expression of CYP7A1, we did not note any changes in the concentration of TBA. it is possible that the non-classical synthesis of bile acid may have occurred independent of SCP2 regulation. As for lecithin, the same changes as in cholesterol were observed in the hepatocyte supernatant. Thus, SCP2 may partly participate in the transport of phospholipids in hepatocytes leading to an increased secretion of lecithin. Therefore, the increase of bile lithogenicity due to an overexpression of SCP2 may be caused by two factors. One is that SCP2 might accelerate the synthesis of cholesterol in liver cells, and the other is that SCP2 may promote the trafficking of cholesterol out of hepatocytes into bile.

In conclusion, SCP2 can affect cholesterol levels, enhance HMGCR expression and repress CYP7A1 expression. Thus it may function as aa moderator for HMGCR in hepatocytes. The results suggest that SCP2 may influence cholesterol metabolism and bile lithogenicity of human liver cells by regulating the expressions of HMGCR and CYP7A1.

## REFERENCES


1. Lammert F, Sauerbruch T. Mechanisms of disease: the genetic epidemiology of gallbladder stones. Nat Clin Pract Gastroenterol Hepatol 2005;2:423-33.
2. Portincasa P, Moschetta A, Palasciano G. Cholesterol gallstone disease. Lancet 2006;368:230-39.
3. Marschall HU, Einarsson C. Gallstone disease. J Intern Med 2007;261:529-42.
4. Venneman NG, van Erpecum KJ. Pathogenesis of gallstones. Gastroenterol Clin North Am 2010;39:171-83.
5. Wirtz KW. Phospholipid transfer proteins revisited. Biochem J 1997;324:353-60.
6. Tsuneoka M, Yamamoto A, Fujiki Y, Tashiro Y. Nonspecific lipid transfer protein (sterol carrier protein-2) is located in rat liver peroxisomes. J Biochem 1988;104:560-4.
7. Keller GA, Scallen TJ, Clarke D, et al. Subcellular localization of sterol carrier protein-2 in rat hepatocytes: its primary localization to peroxisomes. J Cell Biol 1989;108:1353-61.
8. Schroeder F, Butko P, Hapala I, Scallen TJ. Intermembrane cholesterol transfer: role of sterol carrier proteins and phosphatidylserine. Lipids 1990;25:669-74.
9. Kraemer R, Pomerantz KB, Kesav S, et al. holesterol enrichment enhances expression of sterol carrier protein 2:implications for its function in intracellular cholesterol trafficking. J Lipid Res 1995;36:2630-8.
10. Puglielli L, Rigotti A, Amigo L, et al. Modulation of intrahepatic cholesterol trafficking: evidence by in vivo antisense treatment for the involvement of sterol carrier protein 2 in newly synthesized cholesterol transport into rat bile. Biochem J 1996;317:681-7.
11. Noland BJ, Arebalo RE, Hansbury E, Scallen TJ. Purification and properties of sterol carrier protein-2. J Biol Chem 1980;255:4282-9.
12. Amigo L, Zanlungo S, Miquel JF, et al. Hepatic overexpression of sterol carrier protein-2 inhibits VLDL production and reciprocally enhances biliary lipid secretion. J Lipid Res 2003;44:399-407.
13. Mukherji M, Kershaw NJ, Schofield CJ, et al. Utilization of sterol carrier protein 2 by phytanoyl-CoA 2-hydroxylase in the peroxisomal alpha oxidation of phytanic acid. Chem Biol 2002;9:597-605.

14. Seltman H, Diven W, Rizk M, et al. Regulation of bile-acid synthesis. Role of sterol carrier protein 2 in the biosynthesis of 7 alpha-hydroxycholesterol. Biochem J 1985;230:19-24.
15. Lidstrom B, Wikvall K. The role of sterol carrier protein 2 and other hepatic lipid-binding proteins in bile-acid biosynthesis. Biochem J 1986;238:879-8.
16. Stolowich NJ, Petrescu AD, Huang H, et al. Sterol carrier protein 2: structure reveals function. Cell Mol Life Sci 2002;59:193-212.
17. Fuchs M, Hafer A, Münch C, et al. Disruption of the sterol carrier protein 2 gene in mice impairs biliary lipid and hepatic cholesterol metabolism. J Biol Chem 2001;276:48058-65.
18. Cui Y, Li Z, Zhao E, et al. Overexpression of sterol carrier protein 2 in patients with hereditary cholesterol gallstones. BMC Gastroenterology 2011;11:10.
19. Thomas PJ, Hofmann AF. A simple calculation of the lithogenic index of bile: expressing biliary lipid composition on rectangular coordinates. Gastroenterology 1973;65:698-700.
20. Barth CA, Schwarz LR. Transcellular transport of fluorescein in hepatocyte monolayers: Evidence for functional polarity of cells in culture. Proc Natl Acad Sci USA 1982;79:4985-7.
21. Ito T, Kawata S, Imai Y, et al. Hepatic cholesterol metabolism in patients with cholesterol gallstones: Enhanced intracellular transport of cholesterol. Gastroenterology 1996;110:1619-27.
22. Gallegos AM, Atshaves BP, Storey SM, et al. Sterol carrier protein-2 expression alters plasma membrane lipid distribution and cholesterol dynamics. Biochemistry 2001;40: 6493-506.
23. Fuchs M, Lammert F, Wang DQ, et al. Sterol carrier protein 2 participates in hypersecretion of biliary cholesterol during gallstone formation in genetically gallstone-susceptible mice. Biochem J 1998;336:33-7.