Loss of liver FA binding protein significantly alters hepatocyte plasma membrane microdomains

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Abstract Although lipid-rich microdomains of hepatocyte plasma membranes serve as the major scaffolding regions for cholesterol transport proteins important in cholesterol disposition, little is known regarding intracellular factors regulating cholesterol distribution therein. On the basis of its ability to bind cholesterol and alter hepatic cholesterol accumulation, the cytosolic liver type FA binding protein (L-FABP) was hypothesized to be a candidate protein regulating these microdomains. Compared with wild-type hepatocyte plasma membranes, L-FABP gene ablation significantly increased the proportion of cholesterol-rich microdomains. Lack of L-FABP selectively increased cholesterol, phospholipid (especially phosphatidylcholine), and branched-chain FA accumulation in the cholesterol-rich microdomains. These cholesterol-rich microdomains are important, owing to enrichment therein of significant amounts of key transport proteins involved in uptake of cholesterol [SR-B1, ABCA-1, P-glycoprotein (P-gp), sterol carrier binding protein (SCP-2)], FA transport protein (FATP), and glucose transporters 1 and 2 (GLUT1, GLUT2) insulin receptor. L-FABP gene ablation enhanced the concentration of SCP-2, SR-B1, FATP4, and GLUT1 in the cholesterol-poor microdomains, with functional implications in HDL-mediated uptake and efflux of cholesterol. Thus L-FABP gene ablation significantly impacted the proportion of cholesterol-rich versus cholesterol-poor microdomains in the hepatocyte plasma membrane and altered the distribution of lipids and proteins involved in cholesterol uptake therein. —McIntosh, A. L., B. P. Atshaves, S. M. Storey, K. K. Landrock, D. Landrock, G. G. Martin, A. B. Kier, and F. Schroeder. Loss of liver FA binding protein significantly alters hepatocyte plasma membrane microdomains. J. Lipid Res. 2012. 53: 467–480.

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Although the liver plays a key role in cholesterol metabolism and represents the major organ for net removal of cholesterol from the body, mechanisms regulating cholesterol uptake and disposition at the hepatocyte plasma membrane level are only beginning to be understood. Specific lipid-rich microdomains have been identified in hepatocyte plasma membranes not only by biochemical fractionation (1), but also by real-time imaging in living cells (2–4). Uptake of cholesterol, especially that mediated by HDL, is most rapid through cholesterol-rich rather than cholesterol-poor microdomains in living cells as well as in isolated plasma membranes (1, 5–10). Cholesterol is the driving force for lipid-rich microdomain formation in plasma membranes (11), providing a platform for several proteins known to regulate uptake and transport of cholesterol (12–15), FAs (16), and glucose (17–19). The microdomain concept, despite continually evolving details, provides a framework for understanding location and function of receptors, transport/translocase proteins, and downstream signaling molecules. Despite the importance of cholesterol for the very existence of microdomains, little is known regarding the regulation of cholesterol therein, especially in hepatocytes, which have key roles in the net removal of cholesterol from the body as well as in transport and metabolism of both FAs and glucose.

Of the large family of nonenzymatic, cytosolic lipid-binding proteins, liver type FA binding protein (L-FABP) is at the highest concentration, accounting for 3–5% of liver cytosol protein, correlating to a concentration of 100–400 μM (20–22). Although L-FABP’s roles in FA metabolism are...
well established (as reviewed in Refs. 20–23), increasing evidence also suggests a role for L-FABP in intracellular cholesterol trafficking. L-FABP bound two different fluorescent sterol probes, NBD-cholesterol and dansyl-cholesterol, with similar affinity as sterol carrier protein-2 (SCP-2) (24, 25) and L-FABP cross-linked with a photoactivatable cholesterol derivative (3H-FCBP) (24).

Both purified-protein and cultured-cell studies suggested roles for L-FABP in cholesterol transfer from the plasma membrane to intracellular sites. L-FABP selectively enhanced sterol transfer from purified plasma membranes to endoplasmic reticulum (26–28). Chemically blocking the L-FABP ligand binding site inhibited intermembrane sterol transfer (29). L-FABP enhanced microsomal cholesterol esterification of purified proteins and in living cells (28, 30). In cultured cells overexpressing L-FABP, cholesterol uptake, intermembrane transfer, and intracellular cholesterol ester mass were significantly increased (31, 32).

Studies with human genetic variants of L-FABP and with L-FABP gene-ablated mice also support roles in cholesterol as well as FA metabolism. L-FABP gene ablation increased hepatic cholesterol accumulation in mice (24, 33). L-FABP upregulation in SCP-2-/SCP-x-null mice decreased hepatic cholesterol concomitant with biliary hypersecretion of cholesterol (34). In Caucasians, a T94A polymorphism occurs in L-FABP with a frequency of 32–37% (10–13% homozygous) (35–37) and is associated with elevated serum LDL cholesterol and triglycerides, traits associated with increased risk of cardiovascular disease and diabetes mellitus (36, 38). Transfected human Chang liver hepatoma cells overexpressing the L-FABP T94A substitution exhibited cholesterol accumulation (39).

The purpose of the current investigation was to determine the extent to which L-FABP regulates distribution of cholesterol transport proteins in the plasma membrane of cultured primary hepatocytes.

### MATERIALS AND METHODS

**Materials**

Purchase of the Concanavalin-A Sepharose resin was from Pharmacia (Piscataway, NJ); phospholipid standards were from Avanti (Alabaster, AL), whereas neutral lipid and FA standards were from Nu-Chek Prep, Inc. (Elysian, MN). Silica Gel G and 60 TLC plates were obtained from Analtech (Newark, DE) and EM Industries, Inc. (Darmstadt, Germany), respectively. Rabbit polyclonal antisera directed against recombinant human SCP-2 was prepared as described (1). Rabbit polyclonal antisera against caveolin and flotillin were from BD Transduction Laboratories (Palo Alto, CA); rabbit anti-SR-B1 and anti-ABCA-1 were from Novus Biologicals (Littleton, CO). Rabbit anti-Gqα and rabbit anti-SR-B1 and anti-ABCA-1 were from Novus Biologicals (Littleton, CO). Rabbit anti-Gqα was from Sigma (St. Louis, MO). Rabbit anti-CXCR4, GLUT2, and insulin receptor were purchased from Abcam (Cambridge, MA). Rabbit anti-MDR [Pglycoprotein (P-gp)], anti-FATP4, and anti-GLUT1 were from Santa Cruz Bio-technology, Inc. (Santa Cruz, CA). NBD-cholesterol, 22-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl)amin]-23,24-bisnor-5-cholen-3β-ol, was obtained from Invitrogen (Carlsbad, CA), and purified human HDL was purchased from CalBiochem, EMD Biosciences (San Diego, CA). All reagents and solvents used were of the highest grade available and were cell culture tested.

**Animals**

The experiments described herein using mice were approved by the Institutional Animal Care and Use Committee at Texas A and M University. Control wild-type mice (C57BL/6NCr) were obtained at 6 weeks old from the National Cancer Institute (Frederick Cancer Research and Developmental Center, Maryland). L-FABP gene-ablated mice (L-FABP null, /L-FABP) were generated as described previously (40) and backcrossed to C57BL/6NCr mice to the N10 generation as described (24). All mice were kept under a 12 h light/dark cycle in a temperature-controlled facility (25°C) with access to food (standard rodent chow mix, 5% fat calories) and water ad libitum. Monitoring for infectious diseases was performed quarterly, and all mice were free of all known mouse pathogens.

**Hepatocyte isolation and culture**

Livers of 12 week-old male L-FABP-null and wild-type mice were removed, and isolated hepatocytes were cultured as described earlier (17). The cultured primary hepatocytes had an undiminished ability to synthesize and secrete albumin, synthesize and secrete apolipoproteins (e.g., apoA-1 and apoB), secrete lipoproteins (VLDL, HDL), express SCP-2 and L-FABP, take up FA, transport FA within the cell, and oxidize FA for 2–4 days (17, 18). Thus all experiments were performed with hepatocytes cultured for ≤2 days.

**HDL isolation from serum**

HDL was isolated from mouse sera using a 0.5 M CaC6D3TA density gradient ultracentrifugation technique that separates the serum into individual lipoproteins based upon density. The density profile of the gradient was calibrated measuring the refractive index throughout (41). Subsequently, the volume of the HDL subfraction was measured and the HDL protein concentration was determined by a modified Bradford colorimetric assay using the BioRad Dye Reagent and BSA as the standard. The HDL subfraction was then assayed for total cholesterol and free cholesterol using the colorimetric assay kits (Wako Cholesterol E, total cholesterol, catalog no. 439-17501 and Wako Free Cholesterol E, catalog no. 435-35801; Wako Diagnostics, Richmond, VA). The cholesterol ester content was then calculated by subtraction. All colorimetric assays were performed using Costar 96-well microtiter plates utilizing the BioTek Synergy 2 microplate reader (BioTek Instruments; Winooski, VT).

**Confocal laser-scanning microscopy of NBD-cholesterol**

Hepatocytes were cultured as described above and plated (150,000) on collagen-coated 24-well Nunc Lab-Tek chambered coverglasses (VWR, West Chester, PA). For uptake imaging, the cells were washed twice with PBS, 1 ml of PBS added, and placed on an inverted microscope (Zeiss Axiovert 135; Thornwood, NY) with a heated stage (37°C). After 10 min of equilibration, NBD-cholesterol was added in the form of human HDL complexes containing NBD-cholesterol (5 μg/ml) and monitored using a laser-scanning confocal microscope system (MRC-1024MP; Zeiss, Thornwood, NY) at 488 nm, with emission detected using a HQ530/30 filter as described (9). Data were acquired in 1 min time intervals over an hour. For efflux imaging, cells were incubated in culture medium containing 5% FBS and 0.1 μM NBD-cholesterol at 37°C. Hepatocytes were washed twice with PBS and transferred to the heated stage at 37°C. After equilibration for 10 min, unlabeled human HDL in PBS (40 μg/ml) was added and efflux of NBD-cholesterol from the cells was monitored by confocal laser-scanning microscopy (CLSM) for 1 h in 1 min increments (9).

**Cholesterol-rich and cholesterol-poor microdomain isolation**

Purified plasma membranes isolated from primary cultured mouse hepatocytes were simultaneously resolved into

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cholesterol-rich and cholesterol-poor microdomains by a non-
detergent, affinity chromatography method developed by our
laboratory for other cell types (11–14) and modified for hepatocytes (1). Two buffers, buffer A (0.25 M sucrose and 5 mM Tris-
HCL) or buffer B (10 mM HEPES, 140 mM KCL, 1 mM MgCl2,
and 1 mM MnCl2), were used at pH 7.4. This affinity chromatog-
raphy method has been shown to resolve microdomains with less
cross contamination when compared with detergent and high-pH
bicarbonate techniques (1).

**Western blotting analysis of cholesterol-rich and cholesteryl-poor microdomains**

Relative purity of the cholesterol-rich and cholesterol-poor
microdomain fractions was determined using SDS-PAGE fol-
lowed by Western blotting of marker proteins (1, 41): CXCR4 for
cholesterol-poor microdomains; and enrichment in flotillin-1,
SR-B1, Gqα, and GM1 for cholesteryl-rich microdomains. West-
ern blotting of intracellular cholesterol binding proteins L-FABP
and SCP-2 was performed similarly as described previously ex-
cetp that standard curves of recombinant SCP-2 or L-FABP were
included on the gel (1, 41). Relative distribution of intracellular
cholesterol binding proteins L-FABP, SCP-2, other plasma mem-
brane proteins involved in cholesterol transport [ABCA-1 and
MDR (P-gp)], FA transport (FATP-4, GOT), and glucose trans-
port [(GLUT1, GLUT2, insulin receptor (IR)] in cholesteryl-
rich and cholesterol-poor microdomains was determined (1, 41).
Briefly, aliquots of 10 μg protein (homogenate, plasma mem-
brane, cholesterol-rich microdomain, cholesterol-poor microdo-
main) were loaded onto tricine gels (12%), run on a Mini-Protein
II cell (Bio-Rad lab, Hercules, CA) at 100 V constant voltage for
1.5 to 2 h (30 mA per gel initially), and proteins were transferred to
nitrocellulose membranes (Bio-Rad) at 100 V constant voltage
for 2 h. Transferred blots were blocked with 3% gelatin in TBST
(10 mM Tris-HCL, pH 8, 100 mM NaCl, 0.05% Tween-20) for 1
h at room temperature, washed twice with TBST, and incubated
overnight at room temperature with the appropriate dilutions of
polyclonal rabbit primary antibodies in 1% gelatin in TBST: 1:200
(anti-MDR), 1:250 (FATP-4 and anti-SR-B1), 1:500 (anti-ABCA-1,
anti-flotillin, anti-caveolin, anti-SCP-2, anti-CXCR4, and anti-
GLUT1), or 1:1,000 (anti-L-FABP, anti-Gqα, anti-transferrin recep-
tor, and anti-GLUT2) or 1:2,000 (anti-IR). After washing three
times with TBST, blots were incubated for 2 h at room tempera-
ture with a secondary antibody (alkaline-phosphatase conjugates
of goat anti-rabbit IgG) diluted 1:4,500 in 1% gelatin TBST. Blots
were again washed three times with TBST, bands of interest were
visualized with Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/
nitro blue tetrazolium tablets (Sigma), and image was acquired
using a computer workstation (IS-500 system from Alpha Inno-
tech; San Leandro, CA) controlling a single-chip charge coupled
device video camera. Densitometric analysis of image files of
each blot was then performed (mean 8-bit gray scale density)
using NIH Image, available at http://rsbweb.nih.gov/nih-image.
L-FABP and SCP-2 levels were obtained by comparison to stan-
dard curves of known amounts of purified L-FABP and SCP-2
on the same blot. After densitometric analysis for both the
known amounts with standard curve and unknown amounts on
Western blots, quantitation of the image intensities in unknown
amounts was made by comparison to the SCP-2 using the linear
range of the standard curve. Relative protein levels were ex-
pressed as integrated density values for protein quantification
where no source of pure protein was available (flotillin, SR-B1,
CXCR4, Gqα, ABCA-1, and P-gp).

**Lipid extraction and analysis**

Lipids were extracted from homogenate, cholesterol-rich, and
cholesterol-poor fractions followed by resolution into individual
lipid classes and quantitation using appropriate standards as
described (1). Isolated lipids were stored at −80°C under an at-
mosphere of N2 to limit oxidation. The Bradford method was
used to determine protein concentration from the dried protein
extract residue digested overnight in 0.2 M KOH (30). Before
use, all glassware was washed with sulfuric acid-chromate to elimi-
nate lipid contamination. GC-MS was used to determine the FA
composition of the phospholipid fraction isolated from the cho-
lesterol-rich and cholesteryl-poor microdomains as described
(1). Briefly, phospholipids were transesterified by acid to convert
the phospholipid acyl chains to FA methyl esters, which were
then resolved according to chain length and saturation by GC/MS
using a RTX-2330 capillary column (0.25 mm inner diameter ×
30 m; Restek, West Chester, PA) with injector and detector tempera-
tures programmed for 100°C for 1 min and ramped at 10°C/min
to 140°C, then 2°C/min to 220°C, held 1 min, then ramped 20°C/min
to 240°C. FA methyl esters were detected using a Thermo-Finnigan
GC/Trace DSQ single-quadrupole mass spectrometer (Thermo
Electron Corporation; Austin, TX) with electron impact and
chemical ionization sources. Identification of individual peaks
was made by comparison to known FA methyl ester standards pur-
chased from NuChek (Elysian, MN) and referenced against a
known concentration of C15:0-methyl ester added prior to analy-
sis. The lipid concentration was normalized to the total protein
in each respective fraction (e.g., the lipid concentration of the
homogenates was normalized to the total homogenate protein)
and plotted as nmoI/mg of protein using SigmaPlot (Systat Soft-
ware, Inc.; San Jose, CA).

**Statistical analysis**

Unless otherwise stated, all data values were expressed as the
mean ± SEM, n = 3–7. Statistical analysis was performed in Graph-
Pad Prism (San Diego, CA) using a one-way ANOVA and Newman-
Keuls multiple comparison posttest. Differences with P < 0.05
were considered statistically significant.

**RESULTS**

**Hepatocyte cholesterol-rich and cholesterol-poor microdomain protein distribution**

Western blotting of homogenates from cultured primary
hepatocytes of wild-type mice detected both cholesterol-rich
microdomain markers, flotillin (Fig. 1B, E) and Gqα
(Fig. 1C, F), as well as the cholesterol-poor microdomain
marker CXCR4 (Fig. 1D, G). Flotillin and Gqα were enriched
>15-fold and 11-fold, respectively, in cholesterol-rich versus
cholesterol-poor microdomains. Cholesterol-poor microdo-
main were augmented in CXCR4 (Fig. 1D), but rela-
tively deficient in flotillin (Fig. 1B) and Gqα (Fig. 1C).
L-FABP gene ablation had no effect on distribution of the
respective cholesterol-rich (Fig. 1B, C) or cholesterol-poor
(Fig. 1D) microdomain markers.

Although L-FABP gene ablation did not alter the dis-
tribution of these protein markers, the total amount of
cholesterol-rich versus cholesterol-poor microdomains
was significantly increased. The cholesterol-poor and
cholesterol-rich fractions represented 63 ± 6% and 37 ± 6%,
respectively, of plasma membrane protein in wild-type
hepatocytes (Fig. 1A). L-FABP gene ablation significantly
increased the proportion of cholesterol-rich microdomain
protein by 1.4-fold (from 37% to 53%), while concomitantly

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cholesterol-rich domains similarly as in wild-type (Fig. 2A) but the cholesterol mass significantly increased 25% as compared with wild-type (Fig. 2B).

Thus, the distribution of lipid markers GM1, cholesterol, and SM was consistent with that of the protein markers in identifying cholesterol-rich and cholesterol-poor microdomains isolated from cultured primary hepatocytes.

Phospholipid composition

In wild-type hepatocytes, cholesterol-rich microdomains were significantly enriched 2–3-fold in total lipids (Fig. 3A) and phospholipids (Fig. 3B) with an increased cholesterol/phospholipid molar ratio (Fig. 3C) as compared with homogenate. Conversely, the total lipid and the total phospholipid were reduced 2–4-fold in cholesterol-poor
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L-FABP alters plasma membrane microdomains, but the ratio of cholesterol/phospholipid was not significantly different.

L-FABP gene ablation did not alter the total concentration of these phospholipids in the hepatocyte homogenate. However, total phospholipid significantly increased by 22% in cholesterol-rich microdomains (Fig. 3B). Because gene ablation increased both the cholesterol (Fig. 2B) and phospholipid in the cholesterol-rich microdomains, the cholesterol/phospholipid ratio was not significantly altered (Fig. 3C).

Because L-FABP gene ablation significantly increased the phospholipids, individual phospholipid subclasses were examined. The major phospholipid classes in homogenates of wild-type hepatocytes based on both mass (Fig. 4A) and percent composition (Fig. 4B) were: phosphatidylcholine (PC) >> phosphatidylethanolamine, SM, phosphatidylinositol (PI), phosphatidylserine (PS). As compared with cholesterol-poor microdomains (Fig. 4C, D), the cholesterol-rich microdomains were highly enriched, primarily in PC (Fig. 4E, F), and consequently decreased nearly 2-fold in the ratio of anionic/neutral zwitterionic phospholipids: \((PS + PI)/(PC + PE + SM)\) (Fig. 4G, H).

L-FABP gene ablation significantly altered the phospholipid subclasses in cholesterol-rich but not cholesterol-poor microdomains. In general, L-FABP ablation decreased SM and PS mass (Fig. 4A) as well as percent composition (Fig. 4B) in the hepatocyte homogenate. Similar findings were obtained in cholesterol-rich microdomains wherein the decrease in SM mass (Fig. 4E) and percent composition (Fig. 4F) were significant. PC mass (Fig. 4E) and percent composition (Fig. 4F) were both significantly increased in cholesterol-rich microdomains. In contrast, neither amount nor percent composition of phospholipid classes in the cholesterol-poor microdomains was affected. Additionally, L-FABP gene ablation had no significant effect on the ratio of anionic/neutral charged phospholipids in either the cholesterol-poor (Fig. 4G) or cholesterol-rich (Fig. 4H) microdomains.

Thus, L-FABP gene ablation significantly increased the level of total phospholipid by 22% in cholesterol-rich microdomains (Fig. 3B). Ablation also affected select
in the amount of select straight-chain FAs, especially C20:4n-6 (arachidonic acid), which decreased by 45% in homogenates (see supplementary Table I). These and other small changes were also observed based on FA percent composition (see supplementary Table I). Despite these alterations L-FABP gene ablation did not change the overall percent composition of the major groups of straight-chain FAs, including saturated, unsaturated, monounsaturated, and polyunsaturated, as well as their respective ratios (Table 1) in homogenates.

The FA composition of phospholipids from plasma membranes of wild-type hepatocytes followed basically the same order as in the homogenate, except that plasma phospholipid subclasses, especially PC (increased) and SM (decreased), in cholesterol-rich microdomains.

**Straight-chain FA composition of phospholipids**

The major straight-chain FAs in phospholipids of wild-type hepatocytes were in the order 16:0, 18:0 > 18:1n-9, 18:2n-6 > 20:4n-6 > 20:5n-3, 22:6n-3 (see supplementary Table I). Based on FA saturation, the unsaturated (especially polyunsaturated) FAs were more prevalent than the saturated straight-chain FAs, such that the ratios of saturated/unsaturated and polyunsaturated/monounsaturated FAs were 0.88 ± 0.06 and 1.5 ± 0.2, respectively (Table 1). L-FABP gene ablation elicited small but significant changes in the amount of select straight-chain FAs, especially C20:4n-6 (arachidonic acid), which decreased by 45% in homogenates (see supplementary Table I). These and other small changes were also observed based on FA percent composition (see supplementary Table I). Despite these alterations L-FABP gene ablation did not change the overall percent composition of the major groups of straight-chain FAs, including saturated, unsaturated, monounsaturated, and polyunsaturated, as well as their respective ratios (Table 1) in homogenates.

The FA composition of phospholipids from plasma membranes of wild-type hepatocytes followed basically the same order as in the homogenate, except that plasma...
membrane phospholipids were essentially devoid of 20:5n-3 and 22:5n-3 (see supplementary Table 1). There were no major enrichments in the overall percent composition of the major groups of straight-chain FAs in phospholipids (Table 1). L-FABP gene ablation elicited several changes in percent FA composition, especially C18:3n-6 (γ-linolenic acid, doubled) and C22:4n-6 (docosatetraenoic acid, decreased 60%) (see supplementary Table 1). Overall percent composition of the major FA groups, was not significantly affected in their relative proportions in plasma membrane phospholipids (Table 2).

The straight-chain FA phospholipid composition of cholesterol-rich and cholesterol-poor microdomains differed significantly. The cholesterol-microdomain phospholipids were highly enriched in mass of 16:0, 18:0, 18:1, 18:2n-6, 20:4n-6, 22:4n-6 and 22:6n-3 (see supplementary Table I). As a result of these and other changes, the cholesterol-rich fraction was differentially enriched nearly 6-, 4-, 3-, and 5-fold in mass of straight-chain saturated, unsaturated, monounsaturated, and polyunsaturated FAs (Table 2). As a result, the ratios of saturated/unsaturated and polyunsaturated/monounsaturated straight-chain FAs were or trended to be higher in cholesterol-rich microdomains (Table 2). L-FABP gene ablation significantly altered the mass and percent composition of several FA species, especially C18:0 (stearic acid increased 1.5-fold), in these microdomains (see supplementary Table I). As a result, the amount of saturated and unsaturated FAs in cholesterol-rich microdomain phospholipids increased significantly, but without altering the ratios of saturated/unsaturated or polyunsaturated/monounsaturated FAs (Table 2). Although L-FABP gene ablation elicited only small changes in individual FAs of the cholesterol-poor microdomains (see supplementary Table II), the aggregate of these changes increased the proportion of polyunsaturated FAs while decreasing that of monounsaturated FAs, such that ratio of polyunsaturated/monounsaturated FAs was increased (Table 2).

Thus, although the total phospholipid FA composition of hepatocyte plasma membranes was similar to that of the cell homogenate, those in cholesterol-rich microdomains were more highly saturated and polyunsaturated, but less monounsaturated, as compared with cholesterol-poor microdomains. L-FABP gene ablation selectively altered individual FA species in the phospholipids, whereas there was little effect on the ratio of saturated/unsaturated or polyunsaturated/monounsaturated FAs in the phospholipids of cholesterol-rich microdomains. L-FABP gene ablation significantly increased the ratio of polyunsaturated/monounsaturated FAs in the cholesterol-poor microdomains.

### Branched-chain FA composition of phospholipids

The major branched-chain FAs in phospholipids of wild-type hepatocytes were phytanic acid and, nearly 3-fold more, its metabolite, pristanic acid (Table 3). L-FABP gene ablation increased the amount and/or percent composition of these branched-chain FAs in hepatocyte homogenates. Whereas plasma membrane phospholipids tended to be more highly saturated and polyunsaturated, but less monounsaturated, as compared with cholesterol-poor microdomains. L-FABP gene ablation selectively altered individual FA species in the phospholipids, whereas there was little effect on the ratio of saturated/unsaturated or polyunsaturated/monounsaturated FAs in the phospholipids of cholesterol-rich microdomains. L-FABP gene ablation significantly increased the ratio of polyunsaturated/monounsaturated FAs in the cholesterol-poor microdomains.

### Table 1. Effect of L-FABP gene ablation on the FA composition by groups (in nmol/mg and percentage composition) in the homogenate and plasma membrane fractions

| FA Group | Homogenate | Plasma Membrane |
|----------|------------|----------------|
|          | Wild-type  | L-FABP KO      | Wild-type  | L-FABP KO      |
|          | (nmol/mg)  | %              | (nmol/mg)  | %              |
| Saturated| 49 ± 3     | 47 ± 3         | 34 ± 2     | 44 ± 3         |
| Unsaturated| 56 ± 2     | 53 ± 2         | 44 ± 2     | 56 ± 3         |
| MUFA     | 22 ± 2     | 21 ± 2         | 22 ± 2     | 22 ± 3         |
| PUFA     | 34 ± 3     | 32 ± 3         | 27 ± 2     | 35 ± 3         |
| Sat./Unsat.| 0.88 ± 0.06| 0.77 ± 0.06    | 0.79 ± 0.05| 0.77 ± 0.06    |
| PUFA/MUFA| 1.5 ± 0.2  | 1.6 ± 0.2      | 1.3 ± 0.1  | 1.3 ± 0.1      |

Values = mean ± SEM, n = 4–5. See supplementary Table I for composition of individual FAs.

### Table 2. Effect of L-FABP gene ablation on the FA group composition (in nmol/mg and group percentage composition) in the cholesterol-poor and cholesterol-rich fractions

| FA Group | Cholesterol-poor Fraction | Cholesterol-rich Fraction |
|----------|---------------------------|---------------------------|
|          | Wild-type  | L-FABP KO      | Wild-type  | L-FABP KO      |
|          | (nmol/mg)  | %              | (nmol/mg)  | %              |
| Saturated| 13 ± 2     | 39 ± 6         | 14 ± 2     | 37 ± 6         |
| Unsaturated| 20 ± 2     | 61 ± 7         | 24 ± 2     | 63 ± 6         |
| MUFA     | 22 ± 2     | 30 ± 4         | 9 ± 1      | 23 ± 3         |
| PUFA     | 10 ± 2     | 30 ± 6         | 16 ± 2     | 41 ± 6         |
| Sat./Unsat.| 0.66 ± 0.09| 0.59 ± 0.09    | 0.82 ± 0.04| 0.89 ± 0.03    |
| PUFA/MUFA| 1.0 ± 0.1  | 1.9 ± 0.3      | 1.7 ± 0.1  | 1.7 ± 0.09     |

Values = mean ± SEM, n = 4–5. See supplementary Table II for composition of individual FAs.

* Significant difference of L-FABP KO as compared with WT in cholesterol-poor fraction (P < 0.05).

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to have a higher mass and percent composition of phytanic acid as compared with the homogenate, these differences did not achieve statistical significance (Table 3). L-FABP gene ablation decreased the amount, but not percent composition, of phytanic acid in the plasma membrane phospholipids (Table 3).

Branched-chain FAs were differentially distributed in cholesterol-rich and cholesterol-poor microdomains of the hepatocyte plasma membrane. The levels of phytanic acid and pristanic acid were significantly higher in phospholipids of cholesterol-rich than in those of cholesterol-poor microdomains (Table 3). However, because the total amount of phospholipids was higher in cholesterol-rich than cholesterol-poor microdomains (Fig. 3B), the relative phytanic and pristanic acid composition actually comprised 2-fold and 2.5-fold less percent of the phospholipid FAs in cholesterol-rich versus cholesterol-poor microdomains (Table 3). L-FABP gene ablation increased the amount as well as the percent composition of phytanic acid and pristanic acid in cholesterol-rich but not cholesterol-poor microdomain phospholipids (Table 3).

In summary, branched-chain FAs comprised a significant part of phospholipid acyl chains in hepatocyte plasma membrane cholesterol-rich and cholesterol-poor microdomains. Although not increased in plasma membrane relative to homogenate, branched-chain FAs were differentially distributed, accounting for over 2-fold higher proportion (percent) of phospholipid acyl chains in cholesterol-poor versus cholesterol-rich microdomains. L-FABP gene ablation significantly increased the amount and percent contribution of the branched-chain FAs to the acyl chains of phospholipids from cholesterol-rich microdomains.

Distribution of proteins involved in HDL-mediated cholesterol transport

To determine whether these changes in lipid content, especially the higher cholesterol level, in cholesterol-rich microdomains induced by L-FABP gene ablation were associated with altered distribution of proteins involved in HDL-mediated cholesterol transport, Western blotting was performed to examine the distribution and effect of L-FABP gene ablation on intracellular cholesterol binding proteins (L-FABP, SCP-2) and plasma membrane integral membrane proteins [ABCA-1, MDR1 (P-gp), SR-B1] that are involved in reverse cholesterol transport.

The cytosolic cholesterol binding protein L-FABP was 8.7-fold higher in the cholesterol-poor microdomains as compared with the cholesterol-rich microdomains (Fig. 5A, F). SCP-2, which was not increased in plasma membrane microdomains as compared with cell homogenate, was significantly distributed 2.5-fold higher in cholesterol-rich versus cholesterol-poor microdomains of wild-type plasma membranes (Fig. 5B, G). Despite these differential distributions of these proteins, the level of L-FABP (Fig. 5A) and SCP-2 (Fig. 5B) in cholesterol-rich microdomains was nearly equal, near 5 ng/mg protein, in wild-type hepatocytes. L-FABP gene ablation significantly increased SCP-2 by 4.5-fold in cholesterol-poor microdomains while remaining the same in cholesterol-rich microdomains (Fig. 5B).

Key integral plasma membrane proteins involved in reverse cholesterol transport preferentially localized to cholesterol-rich microdomains in both wild-type and L-FABP-null hepatocyte plasma membranes. ABCA-1 preferentially localized to the cholesterol-rich microdomains by 9-fold as compared with cholesterol-poor microdomains and 2-fold and 3-fold for homogenate (Fig. 5C, H). MDR was preferentially enriched 2–3-fold in cholesterol-rich microdomains (Fig. 5D, I), but not in homogenate. Although SR-B1 was much lower in the plasma membrane subfractions than hepatocyte homogenate, probably due to localization in intracellular membrane vesicles (9, 42, 43), SR-B1 was enriched 8-fold in the cholesterol-rich versus cholesterol-poor microdomains of wild-type hepatocyte plasma membranes (Fig. 5E, J) and 2-fold in the L-FABP-null hepatocytes. L-FABP gene ablation increased the localization of SR-B1 by 4.5-fold in the cholesterol-poor microdomains as compared with wild-type without alteration in the cholesterol-rich microdomains (Fig. 5E).

Thus, key cytosolic and plasma membrane integral proteins involved in cholesterol binding/transport were preferentially distributed to cholesterol-rich microdomains of hepatocyte plasma membranes. Although L-FABP preferentially localized to cholesterol-poor microdomains, nevertheless, the amount of L-FABP in cholesterol-rich microdomains was nearly equivalent to that of SCP-2. L-FABP gene ablation significantly increased the quantity of SCP-2 and SR-B1 in cholesterol-poor but not cholesterol-rich microdomains.

### Table 3. Effect of L-FABP gene ablation on the phospholipid branched-chain FA composition (in nmol/mg or percent composition) in cultured primary hepatocytes

| FA Group                  | Wild-type | L-FABP KO |
|---------------------------|-----------|-----------|
|                           | Homogenate| Plasma Membrane | Cholesterol-rich |
|                           | (nmol/mg) | (nmol/mg) | (nmol/mg) | (nmol/mg) | (nmol/mg) | (nmol/mg) |
| Phytanic acid             | 0.40 ± 0.04 | 0.54 ± 0.04 | 0.51 ± 0.05 | 0.39 ± 0.02 | 0.55 ± 0.05 | 0.44 ± 0.03 |
| Pristanic acid            | 1.1 ± 0.1  | 1.1 ± 0.1  | 0.94 ± 0.05 | 0.99 ± 0.06 |
| Phytanic acid             | 0.25 ± 0.01 | 0.27 ± 0.01 | 0.55 ± 0.05 | 1.0 ± 0.1  | 0.57 ± 0.06 |
| Pristanic acid            | 0.52 ± 0.05 | 0.53 ± 0.04 | 1.0 ± 0.1  | 1.6 ± 0.2  | 0.9 ± 0.1  |

Values = mean ± SEM, n = 4–5.

* Significant difference of L-FABP KO as compared with wild-type within each respective fraction (P < 0.05).

** Significant difference of plasma membrane as compared with homogenate in L-FABP KO (P < 0.05).

*** Significant difference of cholesterol-rich as compared with cholesterol-poor in WT (P < 0.05).

**** Significant difference of cholesterol-rich as compared with cholesterol-poor in L-FABP KO (P < 0.05).
L-FABP alters plasma membrane microdomains

To determine whether the effects of L-FABP gene ablation on distribution of proteins involved in HDL-cholesterol trafficking was specific for cholesterol uptake and intracellular transport, Western blotting was performed to examine the distribution and effect of L-FABP gene ablation on proteins involved in FA (FATP-4, GOT) and glucose (GLUT1, GLUT2, IR) transport.

FATP4 and GOT were preferentially enriched in cholesterol-rich microdomains of wild-type hepatocyte plasma membranes. FATP4 (Fig. 6A, F) was dramatically (55-fold) enriched in the cholesterol-rich microdomains as compared with the cholesterol-poor microdomains. In contrast, GOT was nearly equally distributed (Fig. 6B, G). L-FABP gene ablation did not alter the level of either FATP4 (Fig. 6A) or GOT (Fig. 6B) in homogenates. Although gene ablation did not affect the distribution of FATP4 in cholesterol-rich microdomains, that in cholesterol-poor microdomains was increased 7-fold (Fig. 6A, F). In contrast, L-FABP gene ablation did not significantly alter GOT in either microdomain type.

GLUT1, GLUT2, and IR were all preferentially localized in cholesterol-rich microdomains of wild-type hepatocytes. GLUT1 was enriched 5-fold (Fig. 6C, H) and IR by 3-fold (Fig. 6E, J). GLUT2 was not detectable in cholesterol-poor microdomains (Fig. 6D, I). L-FABP gene ablation significantly increased the expression of GLUT2 (Fig. 6D), did not change that of GLUT1 (Fig. 6C), and decreased that of IR (Fig. 6E) in homogenate. L-FABP gene ablation did not significantly alter the distribution of GLUT1 (Fig. 6C) and IR (Fig. 6E) in cholesterol-rich microdomains. In contrast, L-FABP gene ablation increased or trended to increase the distribution of GLUT1 (Fig. 6C) and IR (Fig. 6E), but not GLUT2 (Fig. 6D), in cholesterol-poor microdomains.

Thus, proteins involved in both FA and glucose transport were preferentially localized in cholesterol-rich microdomains.
greatly enhanced in the presence of NBD-cholesterol-labeled HDLs (9). Cultured primary hepatocytes from wild-type and L-FABP-null mice were incubated with NBD-cholesterol-labeled HDL (5 μg/ml) and imaged by CLSM as described in the Methods. In L-FABP-null hepatocytes, NBD-cholesterol was taken up faster and to a greater extent, potentially due to the increase in SCP-2 and SR-B1 in cholesterol-poor domains (Fig. 7A). Similarly, efflux of the NBD-cholesterol to unlabeled HDL began at a faster rate in L-FABP-null hepatocytes before decreasing to a significantly slower rate than wild-type at a transition time of ~20 min (Fig. 7B). Thus, the loss of L-FABP enhanced both HDL-mediated uptake of cholesterol and initial HDL-mediated efflux.

The effect of L-FABP gene ablation upon serum HDL cholesterol content

HDL was isolated from sera obtained from wild-type control and L-FABP gene-ablated mice, and the HDL cholesterol content was assayed using commercially available colorimetric assay kits as described in the Methods. L-FABP gene ablation significantly (P < 0.05) reduced the total HDL cholesterol level from 573 ± 56 nmol/mg protein (+/−) to 394 ± 46 nmol/mg protein (−/−). The free cholesterol, however, was not significantly affected: 118 ± 19 nmol/mg protein (+/−) vs. 131 ± 19/ nmol/mg protein (−/−). Thus, L-FABP gene ablation reduced HDL total cholesterol content but maintained free cholesterol content constant at the expense of esterified cholesterol, which makes up the bulk of the available HDL cholesterol in serum.

DISCUSSION

Both biochemical fractionation (as reviewed in Refs. 12, 44, 45) and real-time imaging (3, 4, 46, 47) revealed the presence of cholesterol-rich and cholesterol-poor microdomains in cell membranes. Cholesterol-rich microdomains in the plasma membranes of both peripheral cells (8, 48–50) and hepatocytes (1) serve as the major scaffolding regions for cholesterol transport proteins important in uptake and disposition of cholesterol. Spontaneous as well as lipoprotein-mediated cholesterol transfer is much faster through cholesterol-rich than cholesterol-poor microdomains (8, 48–50) and hepatocytes (1) serve as the major scaffolding regions for cholesterol transport proteins important in uptake and disposition of cholesterol. Spontaneous as well as lipoprotein-mediated cholesterol transfer is much faster through cholesterol-rich than cholesterol-poor microdomains, both in vitro (1, 5–8, 47, 48, 51, 52) and in living cells (10). Despite these advances, little is known regarding intracellular factors contributing to cholesterol distribution therein, especially in liver hepatocytes, and organ key to uptake (primarily via HDL) and net removal (via biliary excretion) of cholesterol from the body. Hepatocyte cytosol is especially rich in the cholesterol binding/transport proteins SCP-2 and L-FABP (20, 44, 53, 54). Studies with overexpression and gene-ablated mice have shown that both L-FABP (24, 33, 34, 55–57) and SCP-2 (58–60) play important roles in hepatic cholesterol accumulation and biliary cholesterol efflux. Consequently, we hypothesized both SCP-2 and L-FABP to be candidate proteins regulating cholesterol distribution and properties within cholesterol-rich versus cholesterol-poor microdomains in microdomains. L-FABP gene ablation increased the distribution of these proteins in cholesterol-poor microdomains.

**Uptake and efflux of HDL-mediated cholesterol from living cultured primary hepatocytes**

Because L-FABP was preferentially localized in the cholesterol-poor fractions of plasma membranes and the effect of gene ablation of L-FABP was to upregulate the expression of SCP-2 and SR-B1 (an HDL receptor in hepatocytes involved in reverse cholesterol transport) therein, it was important to determine the functional impact upon HDL-mediated cholesterol uptake. As shown previously, the rate of NBD-cholesterol uptake was very slow but was...
SCP-2 is a cholesterol binding protein previously shown by our laboratory to directly interact with several proteins preferentially localized in cholesterol-rich microdomains such as caveolin-1 in nonhepatocytes (64, 65) and SR-B1 in hepatocytes (9). Whether L-FABP competes with SCP-2 for binding to SR-B1 in hepatocyte cholesterol-rich microdomains remains to be resolved. These findings were consistent with plasma membrane cholesterol-rich microdomains serving as scaffolding platforms for both soluble cholesterol binding proteins SCP-2 and L-FABP and integral plasma membrane proteins involved in both lipid and glucose transport in hepatocytes.

L-FABP gene ablation, like SCP-2 gene ablation, significantly increased the proportion of cholesterol-rich microdomains in the plasma membrane (1). In contrast, SCP-2 overexpression in nonhepatocytes did not alter the proportion of cholesterol-rich vs cholesterol-poor microdomains (48). Because hepatocytes are devoid of caveolin-1, these data suggest that SCP-2 and possibly L-FABP interaction with SR-B1 impacts the proportion of cholesterol-rich vs cholesterol-poor microdomains in hepatocytes.

L-FABP gene ablation significantly increased the distribution of several proteins involved in cholesterol, FA, and glucose transport into cholesterol-poor microdomains. L-FABP gene ablation did not alter the level of these proteins in cholesterol-rich microdomains, but instead increased the level of SCP-2, SR-B1, FATP4, and GLUT1 in the cholesterol-poor microdomains. Disruption of cholesterol-rich

hepatocytes. Although a role for SCP-2 in regulating hepatocyte plasma membrane cholesterol-rich and cholesterol-poor microdomains was established by our laboratory (1, 48), the possible contribution of L-FABP was not previously addressed. Resolving individual contributions is important because L-FABP is 10-fold more prevalent than SCP-2 in hepatocyte cytosol and may be upregulated in liver of L-FABP-null mice (34, 61). Although the liver hepatocyte membrane is made up of approximately 85% basolateral and 15% canalicular membrane components (62), primary hepatocyte cultures typically exhibit a reduced proportion of canalicular membrane components (9). Although canalicular rafts have been isolated by established detergent techniques from purified bile canaliculi (63), non-detergent techniques to resolve both rafts and nonrafts from canalicular membranes remain to be developed. Because the basolateral portion of the membrane is principally involved in cholesterol uptake, the present non-detergent isolation technique has proven useful in examining the effects of L-FABP gene ablation on basolateral rafts and nonrafts, further complementing studies of cultured primary hepatocyte basolateral membranes with CLSM (9).

L-FABP was significantly associated with hepatocyte plasma membrane cholesterol-rich microdomains along with other key proteins involved in cholesterol (ABCA1, MDR, SR-B1), FA (FATP4, GOT), and glucose (GLUT1, GLUT2, IR) transport. The total quantity of L-FABP in cholesterol-rich microdomains was equivalent to that of SCP-2. SCP-2 is a cholesterol binding protein previously shown by our laboratory to directly interact with several proteins preferentially localized in cholesterol-rich microdomains such as caveolin-1 in nonhepatocytes (64, 65) and SR-B1 in hepatocytes (9). Whether L-FABP competes with SCP-2 for binding to SR-B1 in hepatocyte cholesterol-rich microdomains remains to be resolved. These findings were consistent with plasma membrane cholesterol-rich microdomains serving as scaffolding platforms for both soluble cholesterol binding proteins SCP-2 and L-FABP and integral plasma membrane proteins involved in both lipid and glucose transport in hepatocytes.

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Fig. 7. Effect of L-FABP gene ablation on the HDL-mediated NBD-cholesterol uptake and efflux. A: Uptake of NBD-cholesterol from labeled human HDL (5 μg/ml) in PBS as imaged using CLSM (Methods) for hepatocytes from wild-type and L-FABP-null mice. Values plotted as mean ± SE, n = 3–6. B: Representative images (A) of hepatocytes from WT and L-FABP KO mice that were acquired using CLSM prior to (t = 0 min) and after (t = 30 min) addition of NBD-cholesterol-labeled HDL. C: Primary mouse hepatocytes were incubated with serum containing NBD-cholesterol, and subsequently, efflux of the fluorescent NBD-cholesterol to human HDL (40 μg/ml) in wild-type and L-FABP-null mouse hepatocytes was measured using CLSM (Methods). Efflux was expressed as percent NBD-cholesterol remaining within the cell. Values plotted as mean ± SE, n = 3–6. D: Representative images (C) of NBD-cholesterol-labeled hepatocytes from WT and L-FABP KO mice that were acquired using CLSM prior to (t = 0 min) and after (t = 30 min) addition of human HDL.
microdomains and/or inappropriate distribution of cholesterol-rich microdomain-prefering proteins to cholesterol-poor microdomains are thought to adversely affect their function (12, 44, 66–68).

L-FABP gene ablation selectively altered the distribution of lipids in these cholesterol-rich versus cholesterol-poor microdomains. The enrichment of cholesterol, GM1, SM, PC, total lipid, total phospholipid, and straight-chain saturated FAs in cholesterol-rich microdomains of wild-type hepatocyte membranes was consistent with earlier findings (1, 48). SM was found to be approximately 28 ± 4 µg/mg protein, which was only slightly larger than a previously reported value of 18.3 ± 4.9 µg/mg protein in plasma membrane rafts isolated from rat hepatocytes using a non-ionic detergent method (63). Our observation of approximately 6-fold enrichment of SM in the cholesterol-rich vs cholesterol-poor-fraction was also in excellent agreement with that in rat hepatocytes wherein SM was enriched ~6-fold in rafts (63). Unique was the finding of small amounts of branched-chain FAs, comprising a higher percent of phospholipid FAs in cholesterol-poor microdomains. The presence of branched-chain FAs is disruptive of model membrane structure, and accumulation is toxic (69–73). Cholesterol-poor microdomains are more fluid/more disordered than cholesterol-rich microdomains (1, 6, 51, 74, 75). Taken together, these findings suggest a structural basis for phospholipids containing branched-chain FAs to pack more readily into cholesterol-poor microdomains of plasma membranes from wild-type hepatocytes. The larger accumulation of branched-chain FAs in cholesterol-rich microdomains of L-FABP-null hepatocytes may be attributed to the fact that both L-FABP and SCP-2 bind branched-chain FAs (76) with high affinity and enhance the uptake/peroxisomal oxidation/detoxification of branched-chain FAs (69–73). In the absence of L-FABP, branched-chain FA oxidation is reduced (69–73), resulting in increased levels of branched-chain FAs available for incorporation into phospholipids, in this case, into cholesterol-rich microdomains. Thus, lack of L-FABP, a protein whose plasma membrane-associated component preferentially localized in cholesterol-poor microdomains, significantly increased the percent content of branched-chain fatty acyl chain phospholipids of the cholesterol-rich but not cholesterol-poor microdomains. This may have contributed to redirecting/increasing the content of SCP-2, SR-B1, FATP4, and GLUT1 in the cholesterol-poor microdomains.

In summary, L-FABP ablation significantly increased the proportion of cholesterol-rich versus cholesterol-poor microdomains in the hepatocyte plasma membrane as well as altered the distribution of lipids and proteins involved in uptake of cholesterol (especially SCP-2, SR-B1), FA (FATP), and glucose (GLUT1) therein. Although L-FABP preferentially localized to cholesterol-poor microdomains and SCP-2 preferentially localized to cholesterol-rich microdomains in wild-type mice, SCP-2 and SR-B1 were preferentially increased in the cholesterol-poor microdomains with the loss of L-FABP. In peripheral cells, redistribution of SR-B1 and caveolin-1 from disrupted cholesterol-rich microdomains reduced HDL cholesterol efflux (12, 67).

Although this suggests that the increased content of SCP-2 and SR-B1 in cholesterol-poor microdomains in L-FABP-null hepatocytes may not be functional in HDL cholesterol dynamics, this is not completely clear. However, L-FABP gene ablation did increase the proportion of plasma membrane cholesterol-rich microdomains without altering the content of SCP-2 and SR-B1 therein, changes that should increase HDL cholesterol uptake and efflux. This was confirmed as indicated by the increased uptake of HDL-mediated cholesterol and enhanced efflux to HDL. Accordingly, L-FABP gene ablation not only enhanced SR-B1 levels in the cholesterol-poor microdomains but also affected mouse serum HDL-cholesterol levels as observed by the corresponding decrease in total HDL-cholesterol. HDL levels have been observed to correlate inversely to SR-B1 expression levels such that in SR-B1 gene-ablated mice, the HDL levels increased, whereas in the SR-B1 overexpression mice, the HDL levels decreased (77). Potentially, the increased compensatory expression of SR-B1, combined with SCP-2 in the cholesterol-poor microdomains resulting from L-FABP gene ablation, may act as a key player in regulation of HDL-cholesterol-mediated uptake and efflux in the liver and subsequently overall HDL-cholesterol levels in serum of mice. Interestingly, owing to concomitant upregulation of L-FABP, ablation of SCP-2 enhanced HDL-mediated cholesterol efflux and biliary cholesterol secretion (9, 34), whereas ablation of both L-FABP and SCP-2 inhibited HDL-mediated cholesterol efflux (9). Conversely, SCP-2 overexpression (has no L-FABP upregulation) enhances hepatic cholesterol accumulation and biliary cholesterol secretion (41, 58, 78, 79). Taken together, these data suggest what synergistic but also distinct roles of L-FABP and SCP-2 in the distribution of lipids and proteins and their functions in plasma membrane cholesterol-rich versus cholesterol-poor microdomains.

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