Ovarian granulosa cells utilize scavenger receptor SR-BI to evade cellular cholesterol homeostatic control for steroid synthesis

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Abstract Cellular cholesterol is known to be under homeostatic control in nonsteroidogenic cells, and this intrigued us to understand how such control works in steroidogenic cells that additionally use cholesterol for steroid hormone synthesis. We employed primary culture of rat ovarian granulosa cells to study how steroidogenic cells adapt to acquire sufficient cholesterol to meet the demand of active steroidogenesis under the stimulation of gonadotropin follicle-stimulating hormone (FSH) and cytokine transforming growth factor (TGF)β1. We found that TGFβ1 potentiated FSH to upregulate scavenger receptor class B member I (SR-BI) and LDL receptor (LDLR), both functional in uptaking cholesterol as $hHDL_3$ and hLDL supplementation enhanced progesterone production, and the effect of each lipoprotein was completely or partially blocked by SR-BI selective inhibitor BLT-1. Uptaken cholesterol could also be stored in lipid droplets. Importantly, LDLR and SR-BI responded to sterol with different sensitivity. Giving cells lipoproteins or 25-hydroxycholesterol downregulated *Ldlr* but not *Scarb1*; *Scarb1* was ultimately downregulated by excessive sterol accumulation under 25-hydroxycholesterol and aminoglutethimide (inhibitor of steroidogenesis) cotreatment. Furthermore, transcription factors sterol regulatory element-binding protein (SREBP)-2 and liver receptor homolog (LRH)-1 crucially mediated *Ldlr* and *Scarb1* differential response to sterol challenge. This study reveals that ovarian granulosa cells retain the cholesterol homeostatic control machinery like nonsteroidogenic cells, although during active steroidogenesis, they utilize SR-BI to evade such feedback control.—Lai, W-A., Y-T. Yeh, M-T. Lee, L-S. Wu, F-C. Ke, and J-J. Hwang. **Ovarian granulosa cells utilize scavenger receptor SR-BI to evade cellular cholesterol homeostatic control for steroid synthesis. *J. Lipid Res.* 2013.** 54: **365–378.**

Supplementary key words scavenger receptor class B member I • LDL receptor • sterol regulatory element-binding protein • liver receptor homolog-1 • follicle-stimulating hormone • transforming growth factor β1 • steroidogenesis

Cholesterol is an essential structural component of mammalian cell membrane and a precursor for the synthesis of steroid hormones and bile acids (1). During ovarian steroid hormone synthesis, cholesterol is first transported into mitochondrial inner membrane facilitated by the steroidogenic acute regulatory protein (StAR), and then converted to the important sex steroid progesterone under sequential actions of the mitochondrial enzyme P450 cholesterol side chain-cleavage enzyme (P450scc) and endoplasmic reticulum enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone could be further enzymatically processed into androgens and estrogens (2). Cellular cholesterol could be derived from the de novo synthesis pathway or from circulating lipoproteins. For steroidogenic cells, lipoproteins are the major source that provides sufficient cholesterol to meet the demand of steroid hormone synthesis (3). Aside from the ubiquitous LDL receptor (LDLR)-mediated endocytic uptake of LDL-cholesterol adapted by most cell types, steroidogenic cells additionally utilize HDL-derived cholesterol through scavenger receptor class B member I

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Abbreviations: 25-OHC, 25-hydroxycholesterol; 3β-HSD, 3β-hydroxysteroid dehydrogenase; ACTH, adrenocorticotropic hormone; AMG, aminoglutethimide; CE, cholesteryl ester; ChIP, chromatin immunoprecipitation; FSH, follicle-stimulating hormone; LDLR, LDL receptor; LRH-1, liver receptor homolog-1; P450scc, P450 cholesterol side chain-cleavage enzyme; SCAP, SREBP cleavage-activating protein; SR-BI, scavenger receptor class B member I; SRE, sterol response element; SREBP, sterol regulatory element-binding protein; StAR, steroidogenic acute regulatory protein; TGFβ1, transforming growth factor β1.

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(SR-BI), which selectively uptakes cholesteryl ester (CE) without internalizing the whole lipoprotein particle (4, 5). Lipoprotein utilization in steroidogenic cells could be different among species. For rodents, HDL is the major circulating lipoprotein and SR-BI serves as the principal lipoprotein receptor in steroidogenic tissues (4, 6–8), whereas for humans, LDL is the major cholesterol-carrying lipoprotein and the role of LDLR and SR-BI is not entirely clear (9). Earlier limited studies suggest that LDL and LDLR participate in human steroidogenesis, as adrenocorticotropic hormone (ACTH)-increased cortisol level was lower in LDLR-deficient homozygous familial hypercholesterolemia patients and LDL-deficient hypobetalipoproteinemia patients compared with normal subjects (10, 11). Additionally, a recent study indicates SR-BI also takes part in human adrenal steroidogenesis, as human subjects with a missense mutation of SR-BI had reduced corticotropin-stimulated adrenal corticosteroid level, and cells with such mutation had defective uptake of HDL-CE (12).

In steroidogenic cells, SR-BI and LDLR are regulated by tropic hormones. Gonadotropins and ACTH increased the expression of SR-BI and LDLR in murine granulosa cells and adrenocortical cells, respectively (8, 13, 14). Treatment with an ovulation-inducing stimulus of follicle-stimulating hormone (FSH) followed by human chorionic gonadotropin (hCG) also increased the expression of LDLR and SR-BI in rhesus macaque granulosa cells (15). Also, luteinized granulosa cells retrieved from women receiving gonadotropin-based ovulatory regimen had increased cholesterol uptake from LDL and HDL to support steroidogenesis (16). Previous studies revealed that in nonsteroidogenic cells, the cellular cholesterol level is under surveillance by the homeostatic control mechanism, with LDLR being a key regulatory target (17). When cells are at a state of sterol insufficiency, the endoplasmic reticulum-bound transcription factors sterol regulatory element-binding proteins (SREBP) are first transported to the Golgi apparatus through the assistance of a sterol-sensing molecule, SREBP cleavage-activating protein (SCAP). Then under the action of Golgi proteases, the N-terminal transcription factor domain of SREBP is released and translocates into the nucleus, and thereby activates transcription of target genes. There are two SREBP genes encoding three proteins, the splice variants SREBP-1a and SREBP-1c, and SREBP-2. SREBP-2 preferentially controls cholesterogenic genes, including LDLR (*Ldlr*) and HMG-CoA reductase (*Hmgcr*), SREBP-1c controls lipogenic genes, and SREBP-1a activates both categories. Also, it has been reported that SR-BI is potentially regulated by SREBP-1a transactivation through sterol response element (SRE)-like sites in its promoter (18). Notably, SR-BI is a known target of liver receptor homolog-1 (LRH-1), a nuclear receptor of the NR5A subfamily that is critically involved in the regulation of cholesterol metabolism, including reverse cholesterol transport, bile acid synthesis, and ovarian steroidogenesis (19). Whether and how the SREBP-mediated cholesterol feedback control mechanism and LRH-1 are involved in the regulation of LDLR and SR-BI in steroidogenic cells is not clear (20).

Pituitary-secreted FSH is the master hormone that promotes ovarian follicle development and granulosa cell differentiation before ovulation (21), and ovarian cytokine transforming growth factor (TGFβ1) plays an important role in facilitating FSH action (22, 23) including enhancement of progesterone production through increased expression of steroidogenic proteins StAR, P450scc, and 3β-HSD (24–27). We were intrigued by how cellular cholesterol is controlled in steroidogenic cells when facing a great demand of cholesterol for active steroidogenic activity. This study, therefore, was initiated to explore how cholesterol entry mediators LDLR and SR-BI are regulated in response to FSH and TGFβ1 stimulation of steroidogenesis in ovarian granulosa cells. Primary culture of granulosa cells from gonadotropin-primed immature rats was used, as both SR-BI and LDLR pathways are active in in vitro culture condition (8, 13). There were two specific aims. The first was to investigate FSH and TGFβ1 regulation of the expression of SR-BI and LDLR and their subcellular localization as well as functional involvement in steroidogenesis. The second aim was to examine sterol responsiveness of the two receptors and the potential involvement of two key transcription factors SREBP-2 and LRH-1.

MATERIALS AND METHODS

Materials

Ovine FSH (oFSH-19-SIAFP) and equine chorionic gonadotropin (eCG) were purchased from the National Institute of Diabetes and Digestive and Kidney Diseases and National Hormone and Peptide Program and Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA). Recombinant human TGFβ1 and mouse monoclonal antibody against LRH-1 were obtained from R&D Systems Inc. (Minneapolis, MN). DMEM/F-12 culture medium was from GIBCO Life Technologies Inc. (Carlsbad, CA). Engelbreth-Holm-Swarm sarcoma tumors-derived (EHS) matrigel, mouse monoclonal antibody against β-actin, Aminoglutethimide, and 25-hydroxycholesterol were from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antibodies against SR-BI and SREBP-2 and monoclonal antibody against LDLR were purchased from Abcam Inc. (Cambridge, UK), Cayman Chemical Co. (Ann Arbor, MI), and Epitomics Inc. (Burlingame, CA), respectively. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and protein G agarose were from Millipore Corp. (Billerica, MA). BLT-1 was from ChemBridge Corp. (San Diego, CA). Moloney murine leukemia virus (MMLV) reverse transcriptase set and Taq DNA polymerase were respectively obtained from Toyobo Biologics Inc. (Osaka, Japan) and Protech Technology Enterprise Co. (Taipei, Taiwan). MG-132 was from Enzo Life Sciences Inc. (Farmingdale, NY).

Animals

Immature Sprague Dawley-derived rats (23–25 days of age) were obtained from the Animal Center at National Yang-Ming University (Taipei, Taiwan). Rats were maintained under controlled temperature (22–25°C) and light conditions (12 h light/12 h dark). Food (Lab Diet from PMI Feeds Inc., St. Louis, MO) and water were available ad libitum. This study was conducted in accordance with the United States National Research Council's *Guide for the*

Care and Use of Laboratory Animals and institutional guidelines, and it was approved by the Institutional Animal Care and Use Committee of National Yang-Ming University.

Cell culture and treatment

Isolation of ovarian granulosa cells from gonadotropin-primed immature rats was performed as previously described (25). In brief, immature rats were injected once subcutaneously with 15 IU of eCG to stimulate the development of multiple follicles to antral follicle stage. Forty-eight hours later, ovarian granulosa cells of mid- to large-sized antral follicles were isolated and seeded into EHS matrigel-coated culture wares in growth medium (DMEM/F-12, 1:1, 2 μg/ml bovine insulin, 0.1% fatty acid-free BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin), and were allowed to attach for 20 h at 37°C, 5% CO_2-95% air. Cultured cells were then incubated in incubation medium (DMEM/F12, 1:1, 0.1% lactalbumin hydrolysate, 100 U/ml penicillin, and 100 μg/ml streptomycin) for an additional 20 h before the beginning of treatment. Cultured granulosa cells at this point and through additional 48 h of cultivation period exhibited a flattened and fibroblastic morphology, while treatment with FSH rapidly stimulated cell rounding that faded after 24 h; on the other hand, combined treatment with FSH and TGFβ1 induced cell rounding phenomenon that lasted to 48 h, and cells were able to revert to fibroblastic shape 24 h after changing to fresh culture medium (28). Biochemical data also showed that TGFβ1 potentiated FSH action to stimulate granulosa cell progesterone production, a differentiation maker that remained low in the control group during 48 h culture period (25–29). Together, these observations indicated that this cell model does not undergo spontaneous luteinization and is ideal for studying regulation of granulosa cell differentiation.

Immunoblotting analysis

Granulosa cells (approximately 6×10^6) were cultured in matrigel-coated 60 mm culture dishes and treated as described in the figure legends. At the end of culturing, cells were extracted with lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA-disodium, 1% NP-40 alternative, 0.25% sodium-deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 5 mM sodium orthovanadate, and 5 mM sodium fluoride) and sonicated using an ultrasonicator (Missonix Inc., Farmingdale, NY). Cell lysates were analyzed for SR-BI, LDLR, SREBP-2, and LRH-1 with β-actin as an internal control. SDS-PAGE analysis was performed with equal protein amount for each sample. After electrophoresis and electroblotting, blots were blocked with 5% fat-free milk and then incubated with primary antibodies. Specific signals were visualized using HRP-conjugated secondary antibodies and enhanced chemiluminescence substrate (PerkinElmer Inc., Waltham, MA). Signals on X-ray films were quantified using a two-dimensional laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

Immunofluorescence analysis

Granulosa cells (approximately 5×10^5) were seeded on matrigel-coated 12 mm cover slips and treated as described in the figure legends. At the end of culturing, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.05% Triton X-100 for 5 min, and then incubated in the blocking reagent (3% BSA and 3% goat serum in PBS) for 1 h. Cells were then incubated with rabbit antibodies against SR-BI, LDLR, or isotypic immunoglobulin, which served as a negative control for 1 h, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for 1 h. The cover slips were mounted on glass slides, viewed under a fluorescence microscope (Olympus BX50, Tokyo, Japan) equipped with a mercury arc lamp, and photographed using SPOT image capture system (Diagnostic Instruments Inc., Sterling Heights, MI). Only the fluorescence emission intensity, exposure time, and signal output gain value could affect the outcome signal intensity. To preclude overexposed images, the system was set to automatically capture the sample image that displayed the most intense fluorescence signal, that is, FSH+TGFβ1-treated cells. Then the exposure time and signal output gain value of this image was used to capture all of the subsequent samples in a single experiment. In this way, the fluorescent images could reflect the actual emission signal for comparison among samples.

Steroidogenic response to lipoprotein supplementation

Human HDL_3 ($hHDL_3$) and human LDL (hLDL) were prepared as previously described (30). In brief, blood samples from healthy donors were centrifuged at 2,000 g for 30 min to obtain sera, which were then supplemented with 3 mM EDTA-disodium and 1 mM phenylmethylsulfonyl fluoride to avoid proteolytic degradation. Lipoproteins were isolated from the sera through sequential floatation ultracentrifugation using KBr solution ($hHDL_3$ at $1.125\ g/cm^3 < \rho < 1.21\ g/cm^3$, and hLDL at $1.019\ g/cm^3 < \rho < 1.063\ g/cm^3$). The isolated lipoprotein fractions were dialyzed against PBS using a centrifugal filter unit with a mw cutoff of 10 kDa (Millipore Corp., Billerica, MA), and then sterilized by passing through a 0.22 μm syringe filter. Protein concentration of the lipoprotein preparations was determined by the Bradford method, and apolipoprotein constitution was confirmed by gel electrophoresis after delipidation with ether and ethanol extraction as previously described (31, 32).

To assay for lipoprotein-supplemented steroidogenesis, granulosa cells (approximately 5.5×10^5) were cultured in matrigel-coated 24-well plates, treated with FSH (10 ng/ml) and/or TGFβ1 (0.5 ng/ml) for 24 h, and then given $hHDL_3$ or hLDL for an additional 3 h. At the end of culturing, progesterone content in the conditioned medium was determined by enzyme immunoassay described below. To understand the potential role of SR-BI in the process, a selective inhibitor BLT-1 was given 1 h prior to lipoprotein administration. Also we determined the progesterone content in lipoprotein preparations; the estimated progesterone content in our highest treatment dose of $hHDL_3$ and hLDL was approximately 20∼40% of that detected in the conditioned media of unstimulated control cells, and it was less than 1% in FSH+TGFβ1-treated group. The progesterone production of granulosa cells was calculated by subtracting the progesterone content in supplemented lipoprotein from that in the conditioned medium sample and then normalizing to the cell number determined by crystal violet assay as previously described (33).

Enzyme immunoassay

Progesterone levels in the conditioned media were measured using enzyme immunoassay as previously described (26, 27). Progesterone antisera and progesterone-conjugated horseradish peroxidase were produced and verified in the laboratory of Dr. Leang-Shin Wu (National Taiwan University, Taipei, Taiwan). In brief, progesterone standards and conditioned medium samples were incubated in the presence of progesterone-conjugated horseradish peroxidase in anti-progesterone antibody-coated 96-well plates for 2 h at room temperature. This was then rinsed off, and peroxidase substrate 2,20-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium (Sigma Chemical Co.) was added and incubated for 2 h. The absorbance of reaction products was measured at 410 nm using a multimode microplate reader (Infinite 200 PRO, Männerdorf, Switzerland).

Fluorescent staining of cellular neutral lipids

Granulosa cells (approximately 5×10^5) grown on cover slips were treated with FSH and/or TGFβ1 for 24 h followed by the addition of $hHDL_3$ (500 μg/ml) or hLDL (100 μg/ml) in the absence or presence of aminoglutethimide (0.5 mM; an inhibitor of P450scc) for an additional 24 h. At the end of culturing, cells were fixed with 4% paraformaldehyde for 15 min, and then stained with 2 μg/ml BODIPY 493/503 (Molecular Probes/Life Technologies Inc., Carlsbad, CA) for 10 min. Cells on cover slips were then mounted on glass slides and photographed as described above.

RT-PCR analysis

Granulosa cells (approximately 2×10^6) were cultured in matrigel-coated 35 mm culture dishes and treated as described in the figure legends. At the end of culturing, cells were extracted with 1 ml of Trizol reagent per 35 mm dish. After adding 200 μl of chloroform, the mixture was centrifuged at 12,000 *g* for 15 min for phase separation. The upper aqueous phase was retained, mixed with isopropanol at 1:1 ratio, allowed to stand at room temperature for 30 min, and then centrifuged at 12,000 *g* for 10 min to precipitate RNA. The pellet was washed with 75% ethanol and then dissolved in ddH_2O containing 0.1% DEPC. RNA concentration of each sample was estimated using ND-1000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE). RNA sample was then reverse-transcribed to cDNA using MMLV reverse-transcriptase according to the manufacturer's protocol. Taq DNA polymerase was used to amplify cDNA for *Scarb1*, *Ldlr*, and *Hmgcr*, with *Actb* used as an internal control. The forward and reverse primer pairs used are listed in **Table 1**. The PCR cycle number used for each gene was within the linear range of amplification. PCR was performed using Aztec PC-816 thermal cycler (Astec Co., Fukuoka, Japan), and the products were analyzed by 1.5% agarose gel electrophoresis with gel containing HealthView nucleic acid stain (Genomics Co., Taipei, Taiwan) and visualized with a UV transilluminator (Quantum ST-1000, Eberhardzell, Germany).

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) analysis was performed as previously described (28) with modifications (34). Granulosa cells (approximately 1.8×10^7) were cultured in matrigel-coated 100 mm culture dishes and treated with FSH plus TGFβ1 for 24 h. At the end of culturing, cells were fixed in 1% formaldehyde for 10 min at room temperature, and the crosslinking reaction was stopped by adding glycine (final 125 mM) and incubating for 5 min. Cells were then rinsed with cold PBS and extracted for chromatin in 200 μl lysis buffer [50 mM Tris-HCl, pH 8.0, containing 10 mM EDTA-disodium, 1% SDS, and a cocktail of protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 5 mM sodium orthovanadate, and 5 mM sodium fluoride)]. This was then sonicated using an ultrasonicator to obtain DNA fragments with sizes in the range of approximately 400–1000 bp. Then the samples were frozen, thawed on ice, and centrifuged at 15,000 *g* for 15 min at 4°C to precipitate SDS. The supernatant was diluted 5-fold with dilution buffer (16.7 mM Tris-HCl, pH 8.0, containing 167 mM NaCl, 1.2 mM EDTA-disodium, 1.1% Triton X-100, 0.01% SDS, and cocktail of protease and phosphatase inhibitors) and precleared by incubating with 50 μl of protein G agarose blocked with 0.1% BSA and 50 μg/ml salmon sperm DNA for 2 h at 4°C. This was centrifuged, and the supernatant was incubated with antibodies overnight at 4°C, followed by another 50 μl of blocked protein G agarose for 6 h. To remove nonspecific binding, the sample was washed sequentially with low-salt wash buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA-disodium, 1% Triton X-100, 0.1% SDS), high-salt wash buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 2 mM EDTA-disodium, 1% Triton X-100, 0.1% SDS), LiCl buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA-disodium, 1% NP-40, 1% sodium deoxycholate), and TE buffer (10 mM Tris-HCl, 1 mM EDTA-disodium, pH 8). The sample was then eluted with 400 μl of elution buffer (0.1 M $NaHCO_3$, 1% SDS). The eluate was then incubated at 65°C in the presence of 0.2 M NaCl for 4 h to reverse crosslinks, and then incubated with 100 μg/ml proteinase K at 50°C for 1 h to remove associated proteins. After phenol/chloroform extraction and ethanol precipitation, the DNA sample was dissolved in TE buffer and analyzed by PCR and agarose gel electrophoresis. The primer pairs used are listed in Table 1.

TABLE 1. Primer pairs used in RT-PCR and ChIP

Gene	Accession Number	Forward and Reverse Primer Pair
RT-PCR		
Scarb1	NM_031541.1	5′-GCGGACCCTATGTCTACAG-3′
		5′-CGAATGCCAATAGTTGACC-3′
Ldlr	NM_175762.2	5′-CGGGCACTGTTTCCGTGGCT-3′
		5′-GCCACCGTTGGGGAGAACCG-3′
Hmgcr	NM_013134.2	5′-GGTGCGAAGTTCCTTAGTGATGC-3′
		5′-GGATGTAGAGGTTGCGTCCTG-3′
Actb	NM_031144.2	5′-CTTGCAGCTCCTCCGTCGCC-3′
		5′-GCACAGTGTGGGTGACCCCG-3′
ChIP		
Scarb1	NC_005111.2	5′-CAGCGTGTGTCTGGGGCCTG-3′
		5′-CCTGCCAATGCCTCCCGCTC-3′
		5′-GGGGAAACACACAGCGGATA-3′
		5′-ATGACAGAAACCGAGCAGCA-3′
Ldlr	NC_005107.2	5′-CAGGGTGTGGAAGGATGTGG-3′
		5′-CGCTGCAAACACAGGATCAC-3′
		5′-CACACAAGCACCCCAAAACC-3′
		5′-CTGAGAACAGGACAGGCTGG-3′

Statistical analysis

Quantitative data are presented as the mean (± SE) and were analyzed by ANOVA and Duncan's multiple-range test at a significance level of 0.05 using the general linear model of the SAS program (SAS Institute Inc., Cary, NC). Differences between two treatment groups were analyzed using the Student *t*-test at a significance level of 0.05.

RESULTS

Pituitary gonadotropin FSH acting together with cytokine TGFβ1 facilitates ovarian granulosa cell differentiation marked by an enhancement of progesterone synthesis (24–27, 29). We were interested in understanding how FSH and TGFβ1 regulate cholesterol availability for active steroidogenesis and how the machinery of cellular cholesterol homeostatic control works in ovarian granulosa cells.

FSH and TGFβ1 regulation of SR-BI and LDLR in steroidogenic granulosa cells

We first investigated the temporal profile of FSH and TGFβ1 regulation of SR-BI and LDLR expression in rat ovarian granulosa cells. Immunoblotting analysis showed

that at 6 and 12 h posttreatment, FSH±TGFβ1 similarly increased SR-BI and LDLR protein levels, with a greater effect on SR-BI (**Fig. 1A**). While TGFβ1 treatment alone had no effect (data not shown), it augmented FSH action and retained high levels of SR-BI and LDLR at 24 and 48 h posttreatment (Fig. 1A). Interestingly, granulosa cells treated with FSH for 48 h still had an SR-BI protein level significantly higher than the control, whereas the LDLR level was lower than the control (Fig. 1A). Next, we employed immunofluorescence analysis to observe subcellular localization of SR-BI and LDLR. After 24 h of treatment, control and TGFβ1-treated cells showed weak immunostaining activity of SR-BI present at the nucleus and cytoplasm, and FSH treatment increased such staining activity in both compartments (Fig. 1B). Treatment with FSH+TGFβ1 further increased SR-BI immunostaining activity, particularly at the plasma membrane protrusion processes and perinuclear region (Fig. 1B). On the other hand, LDLR, regardless of treatment, was predominantly distributed at cytoplasm in punctate patterns (Fig. 1B). Consistent with the result of immunoblotting analysis, the immunostaining activity of SR-BI and LDLR at 24 h posttreatment was more intense in FSH+TGFβ1-treated cells compared with the control and FSH-treated cells. To further understand the contribution of SR-BI and LDLR in cholesterol uptake in steroidogenic granulosa cells, we next assayed for functional activity of the two receptors.

Potential functional role of SR-BI and LDLR in steroidogenic granulosa cells

The cholesterol uptake activity of SR-BI and LDLR in ovarian granulosa cells was investigated by examining FSH and TGFβ1 stimulation of progesterone production under $hHDL_3$ or hLDL supplementation with the combined use of BLT-1 (experimental designs shown in **Figs. 2A** and **3A**). BLT-1 is a small molecule inhibitor that blocks the selective CE uptake activity of SR-BI (35, 36). $hHDL_3$ is rich in apoA-I and devoid of apoB and apoE, and it was reported to have a high affinity for SR-BI but not LDLR (5, 37). hLDL, on the other hand, is a ligand for LDLR and SR-BI (5), and thus, cells could obtain hLDL-derived cholesterol from both pathways.

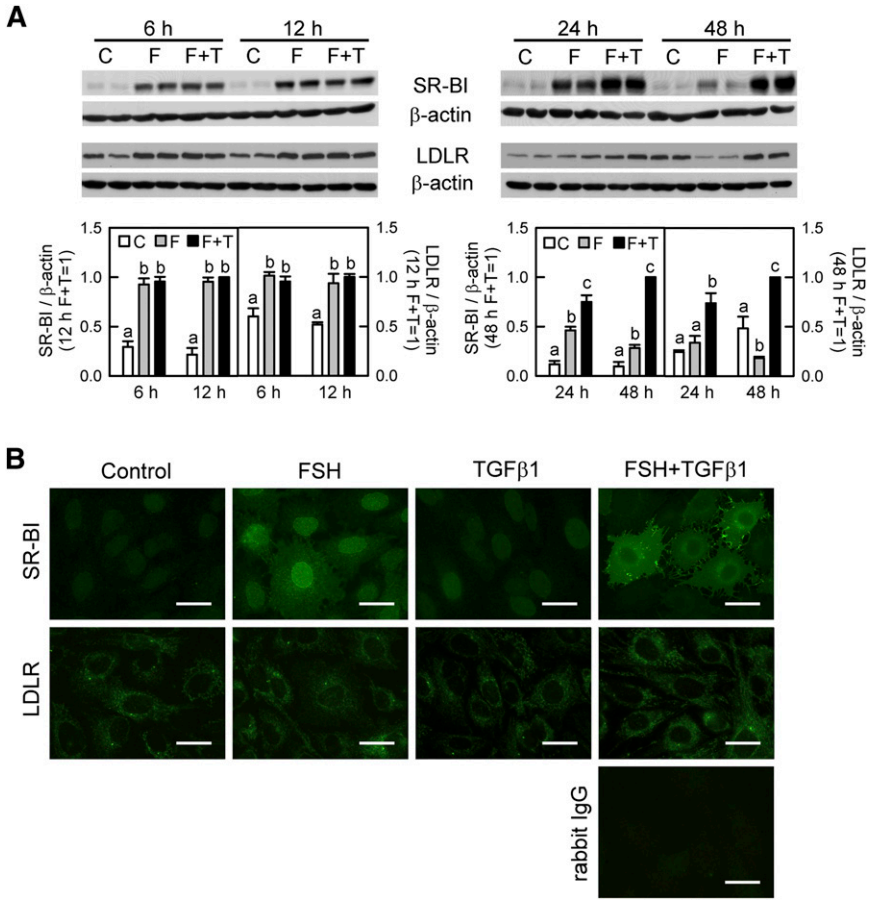

Fig. 1. FSH and TGFβ1 regulation of SR-BI and LDLR protein expression and subcellular localization in steroidogenic granulosa cells. (A) Cultured cells were treated with FSH (10 ng/ml) and/or TGFβ1 (0.5 ng/ml) for various time periods. Cell lysates were prepared and analyzed by immunoblotting for SR-BI and LDLR with β-actin used as an internal control. The relative density ratio was calculated using 12 h or 48 h FSH+TGFβ1-treated value as one. Each bar represents the mean (± SE) relative density (n = 3∼4). Different lowercase letters indicate significant differences among groups in the same treatment period ($P < 0.05$). (B) Cultured cells were treated with FSH and/or TGFβ1 for 24 h, and then analyzed by immunofluorescence for SR-BI and LDLR with normal rabbit IgG used as a specificity control. Bar = 20 μm.

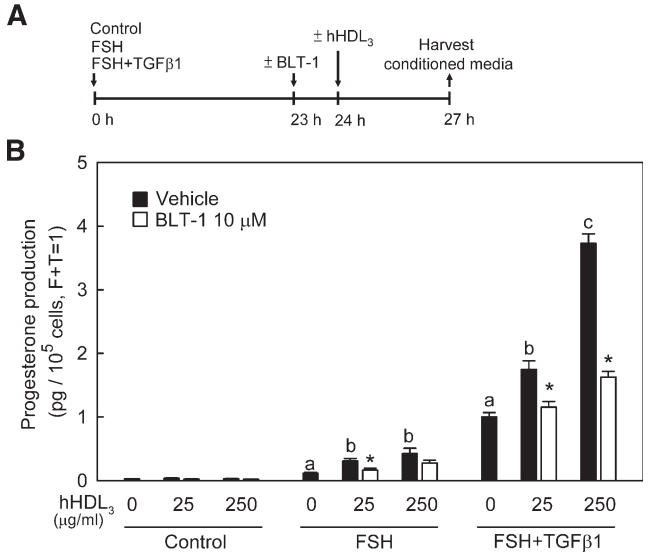

Fig. 2. Effect of BLT-1 on $hHDL_3$ supplementation in FSH and TGFβ1-induced steroidogenesis. (A) Granulosa cells were treated as shown in the experimental protocol. (B) Progesterone levels in conditioned media were determined by enzyme immunoassay and normalized to the cell number. Relative ratio of progesterone production was calculated using the value of FSH+TGFβ1-treated value as one. Each bar represents the mean (± SE) relative progesterone production (n = 6). Different lowercase letters indicate significant differences among groups without BLT-1 treatment in the respective control, FSH, or FSH+TGFβ1 treatment set ($P < 0.05$). Asterisk indicates a significant difference compared with the respective control without BLT-1 treatment ($P < 0.05$).

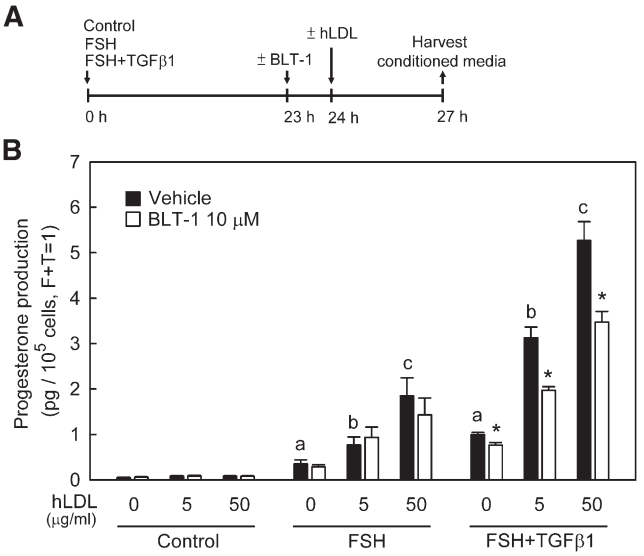

Fig. 3. Effect of BLT-1 on hLDL supplementation in FSH and TGFβ1-induced steroidogenesis. (A) Granulosa cells were treated as shown in the experimental protocol. (B) Progesterone production was determined and calculated as described in Fig. 2. Each bar represents the mean (± SE) progesterone production (n = 6). Different lowercase letters indicate significant differences among groups without BLT-1 treatment in the respective control, FSH, or FSH+TGFβ1 treatment set ($P < 0.05$). Asterisk indicates a significant difference compared with the respective control without BLT-1 treatment ($P < 0.05$).

Granulosa cells were first treated with FSH and TGFβ1 for 23 h to upregulate SR-BI level (control < FSH < FSH+TGFβ1; Fig. 1A) and then treated with or without BLT-1 for an additional hour prior to treatment with $hHDL_3$ for another 3 h (Fig. 2A). Without exogenous lipoprotein supply, TGFβ1 augmented FSH stimulation of progesterone synthesis during the 27 h treatment period (Fig. 2B). Giving cells $hHDL_3$ further increased FSH and TGFβ1-stimulated progesterone production in a dose-dependent manner; this increase was nearly abolished by BLT-1 treatment (Fig. 2B), suggesting that granulosa cells could effectively uptake $hHDL_3$-derived cholesterol through SR-BI for steroidogenesis. We then tested the effect of BLT-1 in hLDL-supplied cells (Fig. 3A). Administration of hLDL dose-dependently increased progesterone production in FSH and TGFβ1-treated cells (Fig. 3B). Noticeably, BLT-1 only partially suppressed hLDL enhancement of FSH+TGFβ1-stimulated progesterone synthesis, whereas BLT-1 had no significant effect on the FSH-treated group (Fig. 3B). This finding implies that granulosa cells could uptake hLDL-derived cholesterol through LDLR and SR-BI routes to meet the high demand of active steroidogenesis.

Uptaken cholesterol could also be stored in the lipid droplet. We next investigated whether FSH and TGFβ1 modulate lipid droplets in granulosa cells using a fluorescent dye BODIPY 493/503 that stains for neutral lipids. Granulosa cells were treated with FSH in the absence or presence of TGFβ1 for 24 h, and then supplemented with lipoproteins for an additional 24 h (**Fig. 4A**). To observe cholesterol accumulation without the complication of differential steroidogenic activity among treatment groups (control, FSH, and FSH+TGFβ1), aminoglutethimide (AMG; an inhibitor of P450scc, the first enzyme in steroidogenesis) was given to cells together with lipoproteins to block steroidogenesis. In the absence of AMG and with lipoprotein supplementation, FSH+TGFβ1 treatment but not FSH alone increased neutral lipid content in cells with diffuse fluorescent staining prominently seen in the cytoplasm, and a few lipid droplets were also present (Fig. 4B, C). Giving cells $hHDL_3$ increased the number of neutral lipid-containing lipid droplets in FSH±TGFβ1-treated cells, and cells supplemented with $hHDL_3$ plus AMG exhibited more prominent increases of lipid droplets in all groups (control, FSH, FSH+TGFβ1) (Fig. 4B), suggesting increased uptake and storage of $hHDL_3$-derived cholesterol. On the other hand, cells given hLDL also displayed increased neutral lipid-staining intensity in all groups (control, FSH, FSH+TGFβ1), and concomitant treatment with hLDL and AMG further enhanced such staining intensity, except in the FSH-treated group (Fig. 4C).

These results together suggest that in ovarian granulosa cells, FSH and TGFβ1-upregulated SR-BI is functional for $hHDL_3$-cholesterol uptake and that hLDL-cholesterol could be internalized through both LDLR and SR-BI pathways; furthermore, cholesterol from both routes could be used for steroid hormone synthesis and stored in lipid droplets.

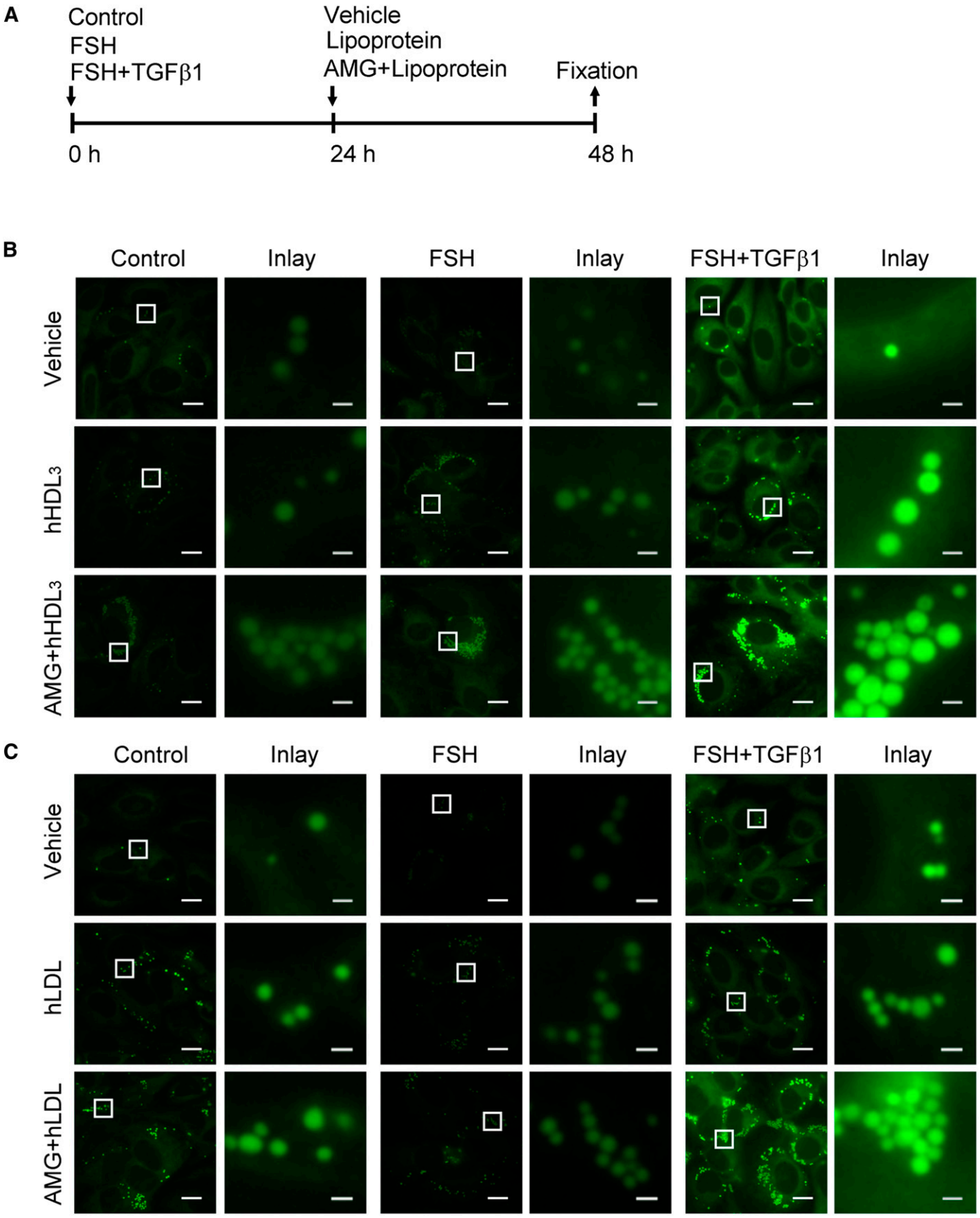

Fig. 4. Fluorescent staining of neutral lipids in steroidogenic granulosa cells. (A) Granulosa cells were treated as shown in the experimental protocol. (B, C) At the end of culturing, cells were fixed and stained with BODIPY 493/503, and then images were taken under a fluorescence microscope. Bar = 10 μm; bar for inlay = 1 μm

Differential sterol responsiveness of SR-BI and LDLR in steroidogenic granulosa cells

We further explored how lipoprotein supplementation modulates the expression of SR-BI gene (*Scarb1*) and two key targets in the cholesterol homeostatic control process, LDLR (*Ldlr*) and HMG-CoA reductase (*Hmgcr*). Granulosa cells were stimulated with FSH and TGFβ1 for 24 h, and then given $hHDL_3$ or hLDL for an additional 3 or 24 h (**Figs. 5A and 6A**). RNA samples were then isolated and analyzed by RT-PCR. It is known in nonsteroidogenic cells that sterol excess leads to downregulation of *Ldlr* and *Hmgcr* (38). We showed that such cellular cholesterol control mechanism functions in steroidogenic granulosa cells as well. Administration of $hHDL_3$ for 3 or 24 h reduced the mRNA levels of *Ldlr* and *Hmgcr* in FSH- and FSH+TGFβ1-treated groups (Fig. 5B). Interestingly, $hHDL_3$ had little effect on *Scarb1* expression in all groups (control, FSH, and FSH+TGFβ1) (Fig. 5B). Administration of hLDL displayed a similar effect to that of $hHDL_3$, except that the control 48 h group (i.e., 24 h post-hLDL supplementation) also had reduced mRNA levels of *Ldlr* and *Hmgcr* (Fig. 6B). These results suggest that the expression of *Scarb1*, unlike *Ldlr* and *Hmgcr*, is relatively insensitive to cholesterol feedback control and, therefore, may be preferentially used in ovarian granulosa cells to uptake and accumulate cholesterol during active steroidogenesis.

Next, we employed a cell-permeable cholesterol analog, 25-hydroxycholesterol (25-OHC) to bypass the different cellular uptake mechanisms for cholesterol, and 25-OHC was also reported to inhibit SREBP transcriptional activity by blocking SCAP-SREBP complex exit from the endoplasmic reticulum (39). Additionally, AMG was used to prevent the diversion of 25-OHC to steroid synthesis and thus could further increase cellular cholesterol accumulation in the presence of 25-OHC. RT-PCR analysis reveals that FSH±TGFβ1 similarly increased the

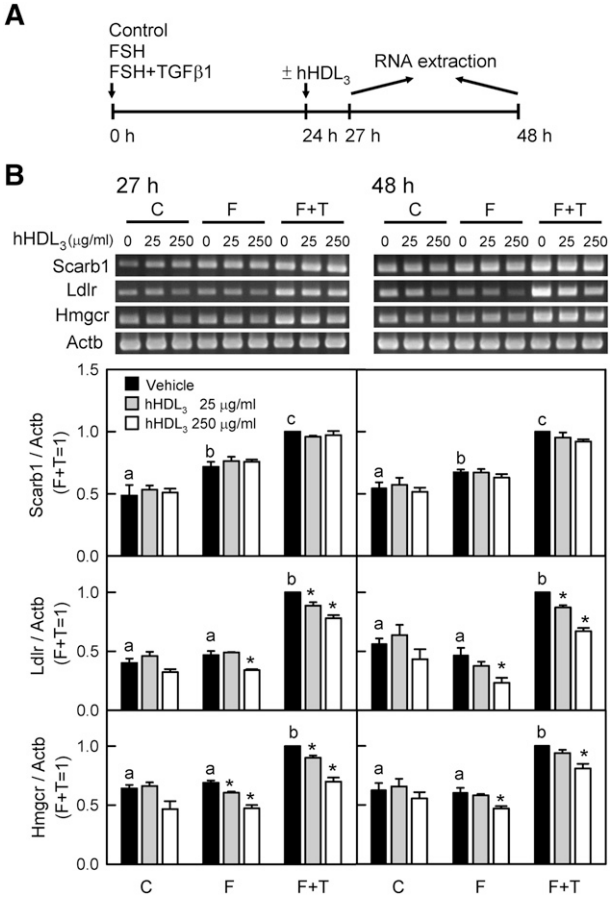

Fig. 5. Effect of $hHDL_3$ supplementation on FSH and TGFβ1-induced expression of cholesterogenic genes in steroidogenic granulosa cells. (A) Cultured cells were treated as shown in the experimental protocol. (B) RNA extracts were analyzed by RT-PCR for *Scarb1*, *Ldlr*, and *Hmgcr* with *Actb* used as an internal control. Relative density ratios were calculated using FSH+TGFβ1-treated value as one at each treatment time period. Each bar represents the mean (± SE) relative density (n = 3). Different lowercase letters indicate significant differences among groups in the absence of lipoprotein supplementation ($P < 0.05$). Asterisk indicates a significant difference compared with respective control without lipoproteins ($P < 0.05$).

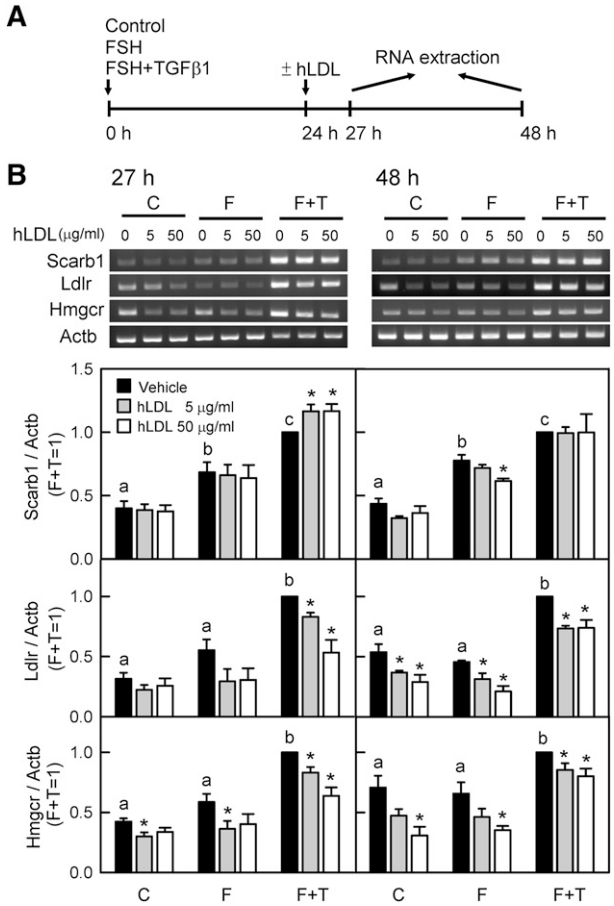

Fig. 6. Effect of hLDL supplementation on FSH and TGFβ1-induced expression of cholesterogenic genes in steroidogenic granulosa cells. (A) Cultured cells were treated as shown in the experimental protocol. (B) RNA extracts were analyzed by RT-PCR, and relative density ratios of *Scarb1*, *Ldlr*, and *Hmgcr* were calculated as described in Fig. 5. Each bar represents the mean (± SE) relative density (n = 3). Different lowercase letters indicate significant differences among groups in the absence of lipoprotein supplementation ($P < 0.05$). Asterisk indicates a significant difference compared with the respective control without lipoproteins ($P < 0.05$).

mRNA levels of *Scarb1*, *Ldlr*, and *Hmgcr* at 4 h posttreatment, and TGFβ1 further enhanced FSH action at 24 h posttreatment (**Fig. 7**). Administration of 25-OHC downregulated the basal and FSH±TGFβ1-increased mRNA levels of *Ldlr* and *Hmgcr* at 4 and 24 h posttreatment, whereas 25-OHC had no effect on *Scarb1* mRNA level (Fig. 7A). Coadministration of 25-OHC and AMG for 4 h had an effect similar to that of 25-OHC alone, and 25-OHC plus AMG administration for 24 h further suppressed FSH±TGFβ1-increased mRNA levels of *Ldlr* and *Hmgcr*, and interestingly, this also attenuated *Scarb1* expression (Fig. 7B).

These results together clearly demonstrate that *Ldlr* and *Hmgcr*, and *Scarb1* genes display differential response to sterol challenge in steroidogenic granulosa cells, and that SREBP may be involved in the process.

Potential role of SREBP and LRH-1 in differential sterol responsiveness of SR-BI and LDLR in steroidogenic granulosa cells

To further identify the underlying mechanisms for differential sterol effect on *Ldlr* and *Scarb1* in ovarian granulosa cells, we focused on two key transcription factors, SREBP-2 and LRH-1. SREBP-2 is the SREBP isoform that preferentially regulates cholesterogenic genes, including *Ldlr* (38). Also, a previous study reported that *Scarb1* promoter contains a proximal SRE and a nuclear receptor NR5A response element (NRE) (18). Additionally, LRH-1 has been known to transactivate *Scarb1* (40), and is specifically expressed in the granulosa cell layer in ovary (41).

We first determined FSH and TGFβ1 regulation of SREBP-2 proteolytic activation using immunoblotting to analyze the full-length and the cleaved mature forms of SREBP-2, respectively designated as SREBP-2(f) and SREBP-2(m). FSH significantly increased the SREBP-2(m) level at 6 and 12 h posttreatment, and TGFβ1 further enhanced FSH effect at 24 and 48 h posttreatment (**Fig. 8A**). Cells given FSH plus TGFβ1 also had an increased level of SREBP-2(f) at 48 h posttreatment (Fig. 8A). Additionally, FSH also increased LRH-1 protein level at 6 and 12 h posttreatment, with the potentiating effect of TGFβ1 lasting 12–48 h posttreatment (Fig. 8B). To confirm SREBP-2 and LRH-1 direct involvement in the expression of *Ldlr* and *Scarb1*, ChIP analysis was used. Cells treated with FSH plus TGFβ1 for 24 h had increased SREBP-2 binding to both

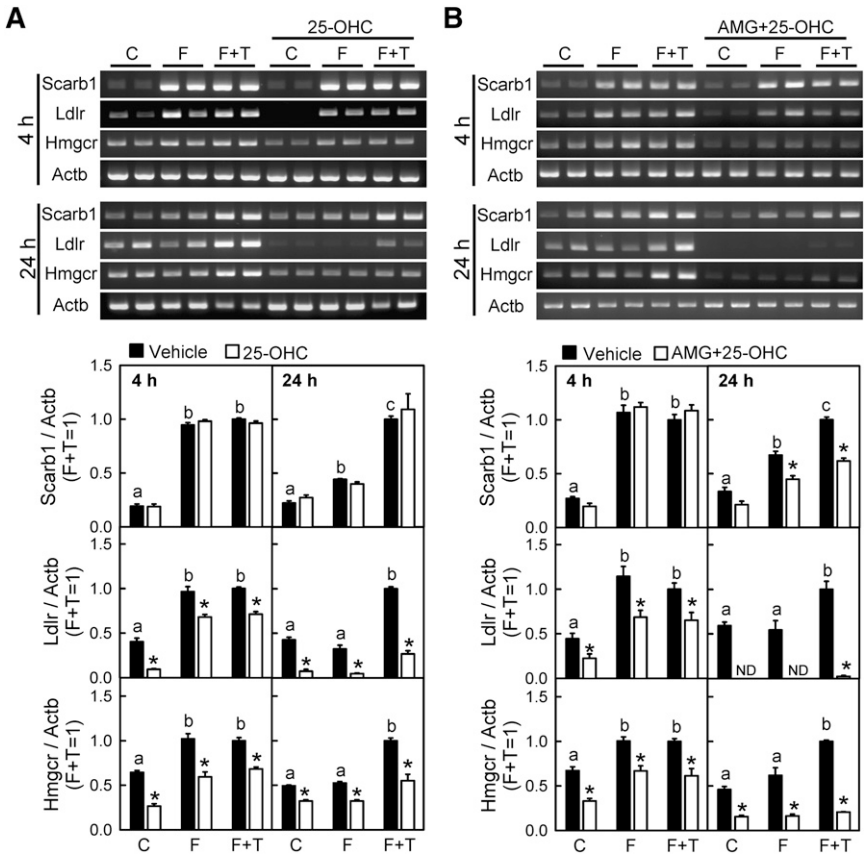

Fig. 7. Effect of 25-OHC and AMG on FSH and TGFβ1-induced expression of cholesterogenic genes in steroidogenic granulosa cells. Cultured cells were pretreated for 1 h with (A) 5 μg/ml 25-OHC or (B) 0.5 mM AMG plus 25-OHC, and then treated with FSH and/or TGFβ1 for additional 4 or 24 h. RNA extracts were analyzed by RT-PCR, and relative density ratios of *Scarb1*, *Ldlr*, and *Hmgcr* were calculated as described in Fig. 5. Each bar represents the mean (± SE) relative density (n = 4). Different lowercase letters indicate significant differences among groups in the absence of 25-OHC±AMG ($P < 0.05$). Asterisk indicates a significant difference compared with the respective control without 25-OHC±AMG ($P < 0.05$). ND indicates density too weak to be accurately quantified.

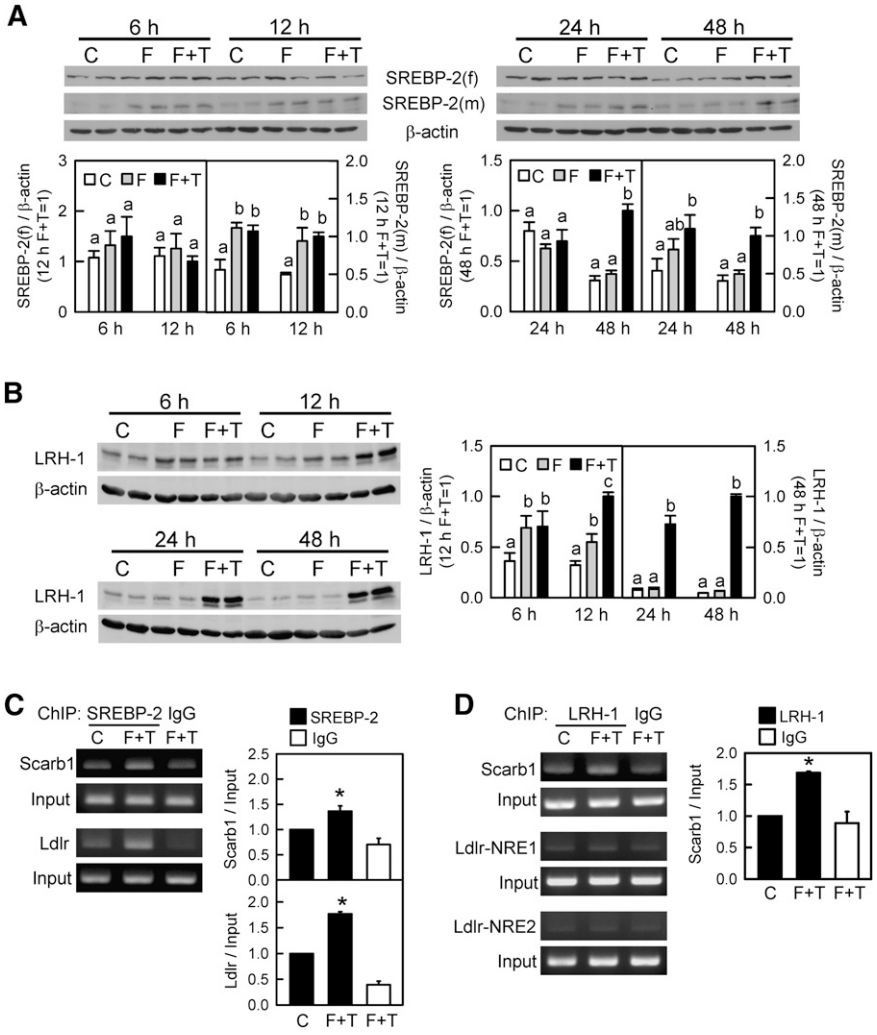

Fig. 8. FSH and TGFβ1 regulation of SREBP-2 and LRH-1 in steroidogenic granulosa cells. (A) Cultured cells were treated with FSH and/or TGFβ1 for various time periods with 10 μM MG-132 added 2 h prior to the end of culturing. Cell lysates were analyzed by immunoblotting for SREBP-2 with β-actin used as an internal control. The full-length and proteolytic mature forms of SREBP-2 are respectively designated as SREBP-2(f) and SREBP-2(m). (B) Cells were treated with FSH and/or TGFβ1, and cell lysates analyzed by immunoblotting for LRH-1. The relative density ratio was calculated using 12 h or 48 h FSH+TGFβ1-treated value as one. Each bar represents the mean (± SE) relative density (n = 4). Different lowercase letters indicate significant differences among groups in the same treatment period ($P < 0.05$). (C, D) Cells were treated with FSH+TGFβ1 for 24 h, and cell lysates were analyzed by ChIP assay using SREBP-2 or LRH-1 antibody with isotypic IgG serving as a negative control. After normalization to input, relative density ratio was calculated with control value as one. Each bar represents the mean (± SE) relative density (n = 3). Asterisk indicates a significant difference compared with the control ($P < 0.05$).

Scarb1 and *Ldlr* promoter regions, with a greater effect on *Ldlr* (Fig. 8C). While *Scarb1* promoter was reported to have an NRE site that could respond to LRH-1 (18, 40), whether LRH-1 regulates *Ldlr* expression is unclear. To identify potential LRH-1 binding sites in *Ldlr* promoter, we analyzed the promoter sequence and found two putative LRH-1 binding sites with only one mismatch from the consensus YCAAGGYCR (where Y = T/C; *R* = G/A) (42) within the range of 2,000 bp upstream of *Ldlr* gene (−173∼−181 and −831∼−839, here designated *Ldlr*-NRE1 and *Ldlr*-NRE2). Therefore, we constructed two primer pairs to encompass each individual site (Table 1). ChIP analysis showed that cells treated with FSH plus TGFβ1 for 24 h had increased LRH-1 binding to *Scarb1* promoter, whereas no obvious LRH-1 binding to NRE1 and NRE2 site in *Ldlr* promoter was detected (Fig. 8D).

We then further investigated the effect of AMG and 25-OHC on SREBP-2 proteolytic activation and LRH-1 protein level in correlation with the changes of LDLR and SR-BI protein levels. Administration with AMG and/or 25-OHC dramatically suppressed the FSH- and FSH+TGFβ1-upregulated protein levels of SREBP-2(m) and LDLR, with 25-OHC exhibiting a stronger effect (**Fig. 9**). Neither AMG nor 25-OHC significantly affected LRH-1 level at 6 h after FSH treatment (Fig. 9). While AMG alone had no effect, 25-OHC significantly decreased the FSH+TGFβ1-upregulated LRH-1 at 24 h posttreatment, and the inhibitory effect was greater under 25-OHC and AMG cotreatment; interestingly, a similar effect

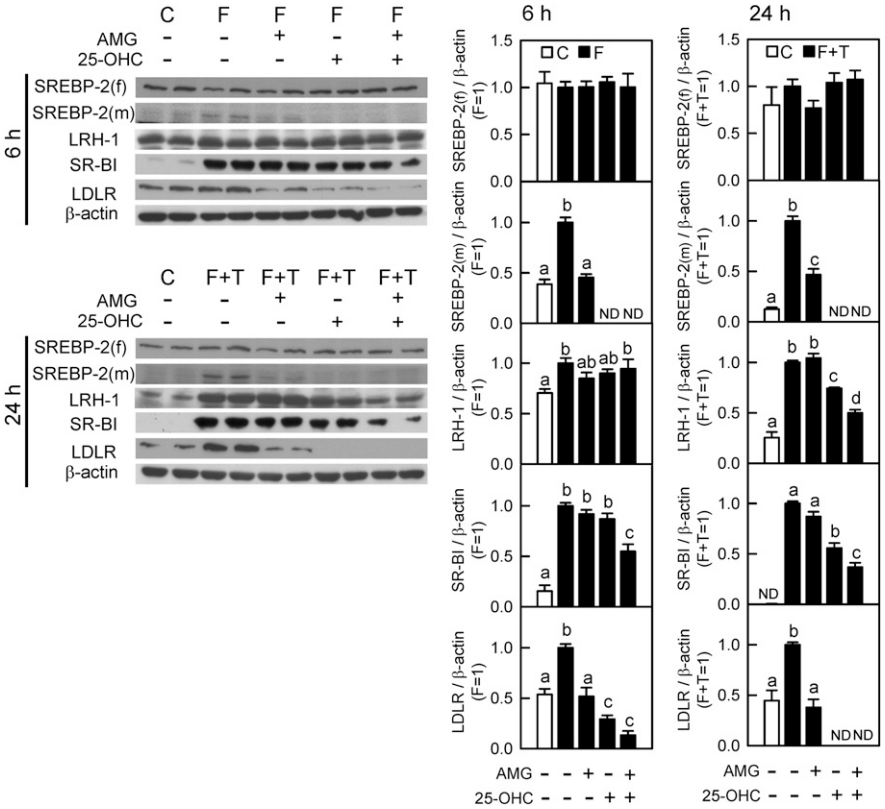

Fig. 9. Effect of 25-OHC and AMG on FSH and TGFβ1 upregulation of lipoprotein receptors and key transcription factors SREBP-2, LRH-1, SR-BI, and LDLR in steroidogenic granulosa cells. Cells were pretreated with 0.5 mM AMG and/or 5 μg/ml 25-OHC for 1 h, and then treated with FSH and/or TGFβ1 for 6 h or 24 h. Then 10 μM MG-132 was added 2 h prior to the end of culturing. Cell lysates were analyzed by immunoblotting for LDLR, SR-BI, SREBP-2, and LRH-1 with β-actin used as an internal control. The relative density ratio was calculated using 6 h FSH-treated or 24 h FSH+TGFβ1-treated value as one. Each bar represents the mean (± SE) relative density (n = 4). Different lowercase letters indicate significant differences among groups in the same treatment period ($P < 0.05$). ND indicates density too weak to be accurately quantified.

was observed on SR-BI protein level (Fig. 9). Altogether, these results indicate SREBP-2 and LRH-1 crucially mediate differential sterol responsiveness of LDLR and SR-BI.

DISCUSSION

Ovarian steroid hormones are essential for female reproductive competence and general well-being, and it is important to understand the control of steroidogenesis, including cellular uptake of the steroid precursor, cholesterol. Although it has been well demonstrated that cellular cholesterol is under homeostatic control in nonsteroidogenic cells (17, 43), how it is regulated in steroidogenic cells remains poorly understood. Using the primary cell culture model system, this study reveals that ovarian granulosa cells retain the cholesterol homeostatic control machinery like nonsteroidogenic cells, although during active steroidogenesis, they utilize SR-BI to evade such control.

Previous studies indicated that lipoprotein receptors SR-BI and LDLR could be upregulated by FSH in ovarian granulosa cells (8, 13). Here, we further provide experimental evidence supporting that TGFβ1 potentiates FSH upregulation of SR-BI and LDLR in ovarian granulosa cells to meet the high demand of cholesterol for active steroidogenic activity. First, treatment with FSH increased protein levels of SR-BI and LDLR, and TGFβ1 could sustain such an increase for at least 48 h posttreatment (Fig. 1). Second, FSH and TGFβ1-upregulated SR-BI and LDLR played active roles as lipoprotein receptors in granulosa cells, as supplying granulosa cells with $hHDL_3$ or hLDL further increased FSH+TGFβ1-stimulated progesterone production and cholesterol accumulation in lipid droplets (Figs. 2–4). $hHDL_3$ was reported to have a high affinity for SR-BI but not LDLR, whereas hLDL is a ligand for LDLR and SR-BI (5, 37). Treatment with BLT-1, a selective inhibitor of SR-BI, almost completely blocked the $hHDL_3$ supplementation-enhanced FSH+TGFβ1-stimulated progesterone production, but it only partially reduced the effect of hLDL (Figs. 2 and 3), suggesting that $hHDL_3$ is largely uptaken through SR-BI, whereas hLDL could be uptaken through both LDLR and SR-BI to enhance progesterone synthesis. Moreover, we found that lipoprotein-derived cholesterol accumulation (reflected by BODIPY 493/503-stained neutral lipids) was apparent and related to the receptor level and that this phenomenon was enhanced when the

steroidogenic process was blocked by the P450scc inhibitor AMG (Fig. 4). In cells given $hHDL_3$±AMG, the staining intensity was related to SR-BI level at 48 h posttreatment (control < FSH<FSH+TGFβ1; Figs. 1A and 4). In cells given hLDL±AMG, the staining intensity was related to the cellular level of LDLR (FSH < control < FSH+TGFβ1; Figs. 1A and 4). Interestingly, we also noticed that, at 24 h posttreatment, FSH-treated cells had an LDLR protein level similar to the control group while having an SR-BI level greater than the control; however, hLDL supplementation-enhanced progesterone production was not affected by BLT-1 pretreatment (Figs. 1A and 3). Although both receptors were reported capable of uptaking cholesterol from LDL (5), our study suggests that hLDL-cholesterol may be more efficiently uptaken through LDLR than SR-BI.

In nonsteroidogenic cells, cellular cholesterol has been clearly demonstrated to be under negative feedback regulation involving transcription factor SREBPs, with *Ldlr* and *Hmgcr* being two canonical SREBP targets (17); whether SREBP regulates SR-BI is unclear. This study identifies that, in steroidogenic granulosa cells, LDLR is also subjected to cholesterol homeostatic control mechanism similar to nonsteroidogenic cells; interestingly, SR-BI responds to sterol challenge differently from LDLR. This is supported by the following lines of evidence. First, supplying granulosa cells lipoproteins downregulated the FSH and TGFβ1-increased expression of *Ldlr* and *Hmgcr* but not *Scarb1* (Figs. 5 and 6). Also, the cell-permeable cholesterol analog 25-OHC exerted a similar effect as lipoproteins (Fig. 7A). Interestingly, we found that granulosa cells given 25-OHC together with AMG (an inhibitor preventing cholesterol conversion to steroids) could also reduce the expression of *Scarb1* at later time and with lesser extent compared with *Ldlr* and *Hmgcr* (Fig. 7B). These results imply that cellular LDLR and SR-BI display different sensitivity to sterol challenge. Second, FSH stimulated SREBP-2 proteolytic maturation with an increased level of SREBP-2(m), and TGFβ1 further enhanced FSH action for a longer duration (Fig. 8A). Additionally, FSH plus TGFβ1

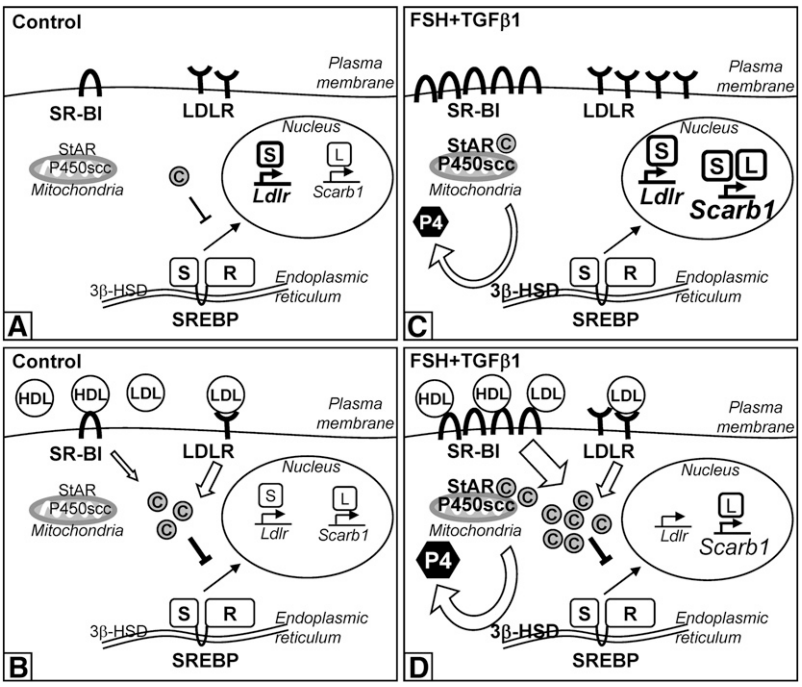

Fig. 10. A proposed working model for FSH and TGFβ1 regulation of cholesterol uptake in steroidogenic granulosa cells. (A) Unstimulated control cells exhibit the presence of LDLR and SR-BI with low steroidogenic activity corresponding to low levels of steroidogenic proteins StAR, P450scc, and 3β-HSD. (B) Unstimulated control cells can uptake cholesterol from lipoproteins. Lipoprotein uptake produces excess cholesterol due to low steroidogenic activity, and this may to some extent repress SREBP activation and the subsequent transcription activation of *Ldlr*. (C) FSH plus TGFβ1 treatment dramatically increases steroidogenic activity (progesterone synthesis) and the associated steroidogenic proteins (StAR, P450scc, and 3β-HSD). This may result in a significant reduction of intracellular cholesterol level, and subsequently induces proteolytic activation of SREBP that in turn transactivates *Ldlr* gene. To meet the high demand of cholesterol for active steroidogenesis, FSH+TGFβ1 treatment additionally upregulates SR-BI through LRH-1, which is less stringently controlled by cholesterol negative feedback to uptake sufficient cholesterol. (D) In the presence of lipoproteins, FSH+TGFβ1-treated cells acquire a greater amount of cholesterol through SR-BI and LDLR, and this further enhances the production of progesterone. An increase of cellular cholesterol rapidly represses SREBP activation and downregulates *Ldlr*, while cholesterol excess ultimately reduces SR-BI level through downregulation of LRH-1. C, cholesterol; L, LRH-1; P4, progesterone; R, regulatory domain of SREBP; S, transcription factor domain of SREBP.

increased SREBP-2 binding to an *Ldlr* promoter fragment containing SRE with moderate binding to *Scarb1* promoter (Fig. 8C). Furthermore, AMG and/or 25-OHC attenuated FSH±TGFβ1-increased SREBP-2(m), and this is parallel to the change of LDLR (Fig. 9). These results suggest that FSH and TGFβ1 upregulation of SREBP-2(m) is likely due to cholesterol shortage consequent to active steroid synthesis and that SREBP-2 predominantly modulates the expression of *Ldlr* and, less so, of *Scarb1*.

Importantly, we identified LRH-1 as a crucial transcription factor that mediates SR-BI response to sterol challenge and that LRH-1 is regulated by FSH and TGFβ1. FSH increased LRH-1 protein level, and TGFβ1 potentiated FSH action (Fig. 8B). Additionally, FSH plus TGFβ1 increased LRH-1 binding to *Scarb1* promoter region containing a putative NRE site, whereas no significant binding was detected on *Ldlr* (Fig. 8D). Moreover, 25-OHC in the absence or presence of AMG reduced the FSH plus TGFβ1-increased LRH-1 at 24 h posttreatment, and this is parallel to the change of SR-BI; whereas no effect was detected at 6 h posttreatment (Fig. 9). This finding indicates that cells, when facing high extent of cholesterol accumulation for long duration, may turn off cholesterol uptake pathways other than LDLR through mechanisms including inhibition of LRH-1 transactivation of *Scarb1*. Granulosa cells given 25-OHC had SREBP-2(m) reduced faster and perhaps at a lower sterol threshold than LRH-1 (Fig. 9), and although SREBP-2 could bind to *Scarb1* promoter (Fig. 8C), it may not be the key player of SR-BI response to sterol challenge. However, in a situation of sterol deficiency, SREBP-2(m) may act with LRH-1 to upregulate *Scarb1*.

A working model is proposed accordingly (**Fig. 10**) to illustrate how steroidogenic granulosa cells adapt to obtain sufficient cholesterol for active steroidogenesis with the conservation of a cholesterol negative feedback control mechanism involving SREBP seen in nonsteroidogenic cells. Under basal condition without tropic hormone stimulation, the LDLR level is monitored by a cellular cholesterol homeostatic control mechanism: SREBP-2-driven expression of LDLR would be downregulated when excess cholesterol is uptaken (Fig. 10A, B). Under FSH and TGFβ1 marked stimulation of progesterone production (upregulation of StAR, P450scc, and 3β-HSD), two mechanistic pathways can be activated but are regulated differentially to meet the great demand of cholesterol in granulosa cells (Fig. 10C, D). During active steroidogenesis, cholesterol conversion into progesterone would cause a decrease in intracellular cholesterol, resulting in proteolytic activation of SREBP-2 that subsequently transactivates *Ldlr*; moreover, FSH+TGFβ1 upregulates LRH-1, which acts with SREBP-2 to augment *Scarb1* expression (Fig. 10C). Cellular cholesterol accumulation rapidly represses SREBP activation and LDLR expression, making FSH+TGFβ1-increased SR-BI the major receptor responsible for bulk entry of cholesterol from HDL and LDL to support steroidogenic need (Fig. 10D). Further increase of the cholesterol level would ultimately reduce the SR-BI level (Fig. 10D), and this might protect cells from cholesterol overload. In all, this study reveals that the cellular cholesterol homeostatic control mechanism is conserved in steroidogenic granulosa cells and that cells evade such control through SR-BI in order to acquire sufficient cholesterol influx for steroid hormone production.

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