Dissertation

Molecular regulation of adrenal function by bile acids

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

Parts of this thesis have been published in the following original paper that is referred to in the text where necessary:

Lei Liu, Katrin Panzitt, Silvia Racedo, Martin Wagner, Wolfgang Platzer, Alex Zaufel, Verena Theiler-Schwetz, Barbara Obermayer-Pietsch, Helmut Müller, Gerald Höfler, Akos Heinemann, Gernot Zollner, Peter Fickert. Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1. Liver International. 2019; doi:10.1111/liv.14052.

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All co-authors declare that they have no conflicts of interest with the content of this thesis and have explicitly agreed to use their data in the thesis.

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Zusammenfassung

Hintergrund und Ziele: Gallensäuren gelten zusätzlich zu ihrer zentralen Funktion in der Fettverdauung als systemisch wirkende Signalmoleküle. Sie sind unter anderem an der Regulation zahlreicher physiologischer Prozesse wie dem Glukose-, Aminosäure- und Lipidmetabolismus beteiligt. Die Gallensäurerezeptoren Farnesoid-X-Rezeptor (FXR), der G-Protein-gekoppelte Gallensäurerezeptor (TGR5) und der Sphingosin-1-phosphatrezeptor 2 (S1PR2) werden auch in Nebennieren exprimiert. Ob Gallensäuren direkt auf Nebennierenrindenzellen wirken und die Steroidsynthese regulieren, ist jedoch noch unklar. Ziel dieser Arbeit war es, die Auswirkungen von Cholestase und insbesondere von Gallensäuren auf die Glucocorticoid-Synthese in den Nebennieren zu untersuchen und die zugrunde liegenden molekularen Mechanismen in adrenocorticalen Zellen aufzuklären.

Methoden: FXR und TGR5 knock-out C57BL/6-Mäuse und Wildtyp Tiere wurden einer Gallengangligatur (common bile duct ligation, CBDL) unterzogen oder mit einer 1% igen Chenodeoxycholsäure (CDCA) Diät gefüttert, um Cholestase zu modellieren. Die humane Nebennierenrindenkarzinomzelllinie H295R wurde mit verschiedenen Gallensäuren inkubiert. Zusätzlich wurden verschiede Signalwege durch spezifische Antagonisten gehemmt. siRNA wurde zur genetischen Hemmung eingesetzt. Einflüsse von Gallensäuren auf die Steroidsynthese wurden durch serologische Tests, Realtime-PCR (RT-PCR) und Western Blotting-Assays bewertet. Die Transaktivierungsaktivität von durch Gallensäuren beeinflussten Transkriptionsfaktoren wurde mittels Luciferase-Aktivitätsassays untersucht.

Ergebnisse: Wir fanden, dass sowohl CBDL Tiere als auch mit CDCA gefütterte Mäuse signifikant erhöhte Spiegel von Corticosteron (das Hauptglucocorticoid der Maus, dem menschlichen Cortisol entsprechend) aufwiesen. Dieses Ergebnis war unabhängig von FXR und TGR5. Ebenso wurde die mRNA- und Proteinexpression der meisten Enzyme der Steroidsynthese unabhängig von diesen beiden

Rezeptoren durch Cholestase und Gallensäuren gesteigert .Die Genexpression von Proteinen, die im Transport und der Neusynthese von Cholesterin in der Nebenniere involviert sind, war nach CBDL und CDCA Fütterung erhöht, während die Cholesterylester-Konzentrationen in den Nebennieren dieser Mäusen abnahmen. In Zellkultur-Assays konnte die Cortisolsekretion von H295R-Zellen durch Taurin-konjugierte CDCA (TCDCA) gesteigert werden. Die mRNA- und Proteinexpression von Enzymen der Steroidsynthese war nach CBDL und TCDCA-Behandlung ebenfalls erhöht. FXR- und TGR5-Agonisten konnten die Cortisolproduktion in H295R-Zellen nicht aktivieren. TCDCA steigerte die Phosphorylierung von extracellular signal-regulated Kinase (ERK) in H295R-Zellen und die Hemmung von ERK reduzierte die Steroidproduktion. JTE-013, ein spezifischer S1PR2 Inhibitor, konnte die durch TCDCA verursachte Phosphorylierung von ERK, die Cortisolsekretion und die Überexpression der Enzyme der Steroidsynthese in H295R-Zellen aufheben. In ähnlicher Weise inhibierte S1PR2 siRNA die Phosphorylierung von ERK und führte zu einer verringerten Cortisolsekretion in H295R-Zellen. Luciferase Assays zeigten eine gesteigerte transkriptionelle Aktivität des Steroidogenic Factor-1 (SF-1) nach TCDCA-Behandlung in H295R-Zellen. Die Zugabe eines inversen SF-1-Agonisten verringerte die durch TCDCA gesteigerte Cortisol Produktion. SF-1 Protein wurde durch pharmakologische Hemmung von S1PR2 und der ERK-Phosphorylierung in H295R-Zellen verringert.

Schlussfolgerungen: Unsere experimentellen Ergebnisse *in vivo* und *in vitro* zeigen, dass supraphysiologische Konzentrationen von Gallensäuren die Steroidsynthese in adrenocortikalen Zellen über einen S1PR2-ERK-SF-1-Signalweg direkt stimulieren können. Diese Erkenntnisse könnten helfen, die Mechanismen von Begleiterscheinungen von Lebererkrankungen und Cholestase wie das Hepatoadrenale Syndrom und Osteoporose, zu verstehen.

Abstract

Background and Aims: Bile acids, the main constituents of bile exclusively secreted by the liver, are considered as signaling molecules except for their function of facilitating digestion and absorption. They are involved in the regulation of multiple physiological activities including glucose, amino acid and lipid metabolism. Some known bile acid binding receptors such as the farnesoid X receptor (FXR), the G protein-coupled bile acid receptor (TGR5), and the sphingosine-1-phosphate receptor 2 (S1PR2) are also found to be expressed in adrenals. However, whether bile acids directly act on adrenocortical cells and regulate steroidogenesis is still unclear. In this thesis, we aimed to study the effects of cholestasis and more specifically of the bile acids on the glucocorticoid synthesis of adrenal glands, and to unravel the underlying molecular mechanisms of regulation of bile acids on steroidogenesis in adrenocortical cells.

Methods: FXR and TGR5 knock out C57BL/6 mice, together with their corresponding wild type littermates were subjected to common bile duct ligation (CBDL) or 1% chenodeoxycholic acid (CDCA) supplemented diet to model cholestasis. Human adrenocortical carcinoma cell line H295R were cultured with various bile acids agonists or antagonists of engaged pathways were applied. siRNA interference was conducted for genetic inhibition when necessary. Systematic influences of bile acids on steroidogenesis were evaluated by serological tests, real time PCR (RT-PCR) and Western Blotting assays. Transactivation activity of transcription factors affected by bile acids was studied by luciferase activity assay.

Results: We found that both CBDL and CDCA-fed mice had significantly elevated levels of corticosterone, the main glucocorticoid in rodent, which is equivalent to human cortisol, in an FXR and TGR5 independent manner. mRNA and protein levels of most steroidogenesis-related enzymes were also increased by CBDL and CDCA feeding independent of FXR and TGR5. In addition, genes related to transport and

de novo synthesis of cholesterol in adrenal gland were found to be increased by CBDL and CDCA feeding as well, while cholesteryl ester concentrations in adrenal glands of CBDL and CDCA-fed mice were strikingly decreased. In cell culture assays, cortisol secretion of H295R cells was substantially enhanced by conjugated CDCA, especially taurine-conjugated CDCA (TCDCA). mRNA and protein expressions of steroidogenesis-related enzymes were also increased upon TCDCA treatment. In contrast, FXR and TGR5 agonists failed to activate cortisol level secreted by H295R cells. Extracellular signal-regulated kinase (ERK) phosphorylation was dramatically elevated by TCDCA treatment and was indispensable for TCDCA-induced steroidogenesis in H295R cells. Application of JTE-013, a specific pharmacological inhibitor of S1PR2 successfully attenuated phosphorylation of ERK and abrogated cortisol secretion caused by TCDCA in H295R cells. As a result, enhanced expressions of steroidogenesis-related genes by TCDCA were also abolished by JTE-013. Likewise, siRNA interference significantly inhibited the mRNA and protein levels of S1PR2, thus leading to lower phosphorylation level of ERK, decreased cortisol secretion and reduced mRNA levels of steroidogenesis-related genes in H295R cells. Luciferase activity assays demonstrated that steroidogenic factor-1 (SF-1) transactivation activity was increased upon TCDCA treatment in H295R cells. Accordingly, addition of a SF-1 inverse agonist also decreased steroidogenesis caused by TCDCA. Protein level of SF-1 was found to be decreased by pharmacological inhibition of S1PR2 and ERK phosphorylation in H295R cells.

Conclusions: Our combined *in vivo* and *in vitro* experimental results indicate that supraphysiological bile acids directly stimulate steroidogenesis in adrenocortical cells via an S1PR2-ERK-SF-1 signaling pathway. These findings help to understand the mechanisms of some diseases such as hepato-adrenal syndrome and osteoporosis encountered in cholestasis.

Abbreviations and definitions

ABCA1	ATP binding cassette subfamily A member 1
ACTH	adrenocorticotropic hormone
ASBT	apical sodium bile acid transporter
BSA	bovine serum albumin
CA	cholic acid
cAMP	cyclic adenosine monophosphate
CBDL	common bile duct ligation
CBG	corticosteroid-binding globulin
CDCA	chenodeoxycholic acid
CRH	corticotropin releasing hormone
CYP11B	cytochrome P450 11B，also referred as steroid 11β-hydroxylase
CYP21	cytochrome P450 family 21, also referred to as steroid 21-hydroxylase
CYP51	lanosterol 14 alpha-demethylase
DCA	deoxycholic acid
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
FXR	farnesoid X receptor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G protein coupled receptors
GR	glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal
HSD3B	3-beta-hydroxysteroid dehydrogenase
HSL	hormone-sensitive lipase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate-buffered saline
PKA	protein kinase A

POMC	pro-opiomelanocortin
RAI	relative adrenal insufficiency
RIPA	radioimmunoprecipitation assay buffer
RXR	retinoid X receptor
S1PR2	sphingosine-1-phosphate receptor 2
SF-1	steroidogenic factor 1
SR-B1	scavenger receptor class B type 1
STAR	steroidogenic acute regulatory protein
TCA	taurocholic acid
TCDCA	taurochenodeoxycholic acid
TGR5	transmembrane G protein-coupled receptor
THCA	taurohyocholic acid
THDCA	taurohyodeoxycholic acid
THE	tetrahydrocortisone
THF	tetrahydrocortisol
TMDCA	tauromurideoxycholic acid
VDR	vitamin D receptor

1. Introduction

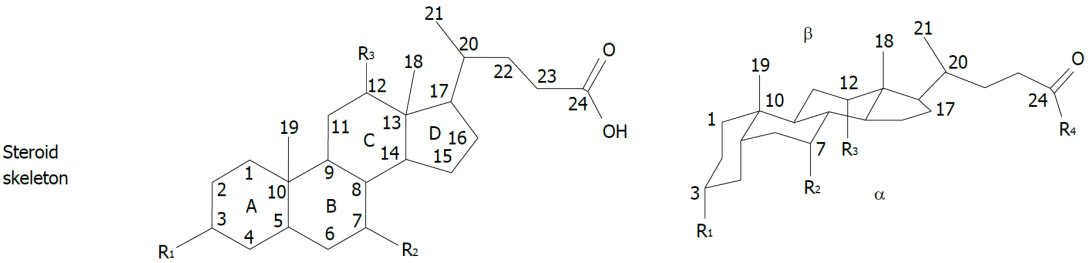
1.1. Bile acids and signaling

1.1.1 Bile acids: structure, metabolism, regulation, and biological functions

Bile acids are a heterogeneous family of complex molecules and actively secreted into bile, together with many endogenous solid constituents such as cholesterol, amino acids, and phospholipids (1). Chemical structure of bile acids was elucidated in 1930s. A numbering system of bile acids was then established and has been used ever since. Figure 1 depicted the carbon skeleton of bile acids with 24 carbon atoms (called C_{24} bile acids) and structures of some abundant bile acids in human bile. Three 6-member rings (A, B, C) and one 5-member ring (D) compose the steroid nucleus. Hydroxyl groups of bile acids face the hydrophilic α-side while the carboxyl groups face the opposing hydrophobic β-side, which make bile acids amphipathic molecules with a great surface activity (2). Bile acids are mainly conjugated with taurine or glycine in human livers. In contrast, in mice, bile acids are mainly conjugated with taurine. As a result of conjugation, bile acids' solubility in aqueous solutions is increased and the sodium and potassium salts form bile salts.

Human bile acids pool is composed of primary bile acids chenodeoxycholic acid (CDCA) and cholic acid (CA), and the secondary bile acids lithocholic acid (LCA) and deoxycholic acid (DCA). In the liver, the primary bile acids are synthesized from cholesterol. Gut bacteria then further convert primary bile acids into secondary bile acids. Figure 2 is a schematic representation covering the main enzymes and intermediates during bile acid synthesis. There are two biosynthesis pathways in the liver: the neutral pathway (the classic pathway that is perceived to be the major pathway) and the acidic pathway (the alternative pathway) (3). The main differences between the two pathways lie in the order of steroid ring modification and side chain cleavage. In the neutral pathway, the rate-limiting enzyme, cholesterol 7α-hydroxylase (CYP7A1), initiates the whole pathway by converting cholesterol into

7α-hydroxycholesterol. Microsomal sterol 12α-hydroxylase (CYP8B1) mediates the hydroxylation of C_4 and is indispensable for CA synthesis. CDCA synthesis, however, does not require CYP8B1. In contrast, sterol 27-hydroxylase (CYP27A1) initiates the acidic pathway. Oxysterol 7α-hydroxylase (CYP7B1) continues the synthesis pathway by catalyzing hydroxylation reaction of cholesterol. Although the acidic pathway typically only produces less than 10% of total bile acids in humans (4), it may be important for patients with liver diseases



Name	R_1	R_2	R_3	R_4
Cholanic acid	H	H	H	OH
Cholic acid	OH	OH	OH	OH
Chenodeoxycholic acid	OH (α)	OH (α)	H	OH
Deoxycholic acid	OH	OH	OH	OH
Ursodeoxycholic acid	OH (α)	OH (β)	H	OH
Lithocholic acid	OH	H	H	OH
Glicocholate	OH	OH	OH	$NHCH_2COO^-$
Taurocholate	OH	OH	OH	$NHCH_2CH_2SO_3^-$

Figure 1 Structures of different species of bile acids in human beings

Groups and conjugations on the carbon skeleton are indicated.

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The majority of bile acids, once synthesized, will circulate between the liver and intestine tract, called enterohepatic circulation. Bile acids synthesized by hepatocytes are secreted into bile and stored in gallbladder. Upon simulation of cholecystokinin secreted from the intestine, the bile acids are released into the

intestine tract. There, most bile acids are reabsorbed by the ileum and transported back to the liver. Such process is typically cycled 4-12 time per day. Bile acids, which spill over into the systemic circulation, will be reabsorbed by the kidney. Only a very small portion of total bile acids is secreted into feces. This part will be replenished by *de novo* synthesis by the liver.

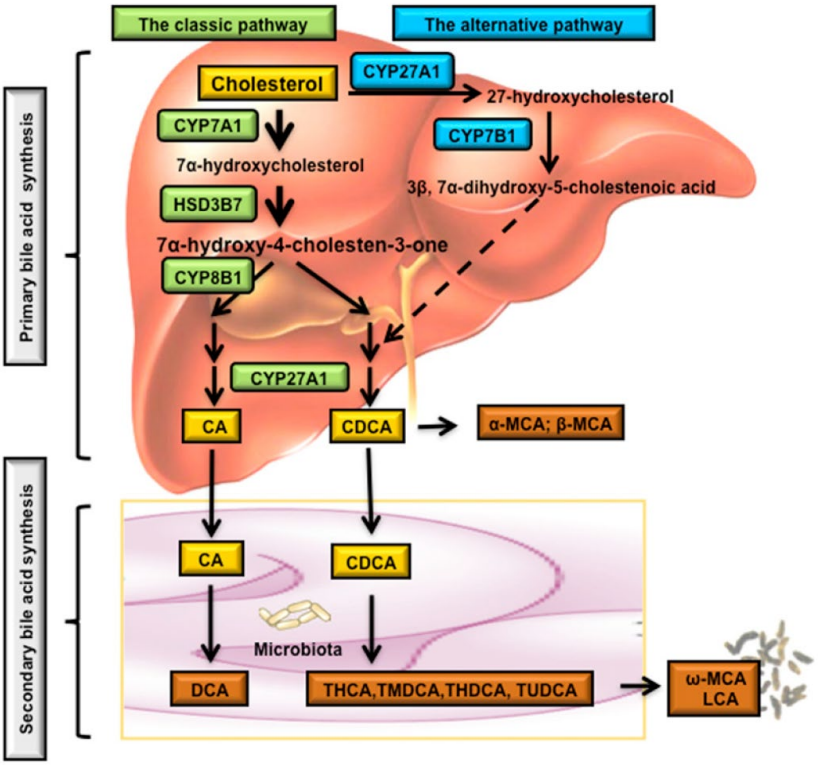

Figure 2 Bile acid biosynthetic pathways in the liver and guts

Two biosynthetic pathways, the classic pathway and alternative pathway, are presented in the schematic. The main difference between the two pathways lies in the fact that cholesterol is converted into 7α-hydroxycholesterol by CYP7A1 and finally used to synthetize cholic acid (CA) and chenodeoxycholic acid (CDCA) in the classic pathways. While in the alternative pathway, the cholesterol is first converted into 27-hydroxycholesterol by CYP27A1 and finally converted into CDCA. In the guts, bacteria convert the primary bile acids (CA and CDCA) into secondary bile acids. In mouse liver, α- and β-MCA is further synthetized from CA and CDCA. DCA, deoxycholic acid; LCA, lithocholic acid; THCA, taurohyocholic acid; THDCA, taurohyodeoxycholic acid; TMDCA, tauromurideoxycholic

acid.

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The physiological regulation of bile acids is mainly dependent on hepatic CYP7A1 enzyme activity by a negative feedback regulation and in a transcriptional manner (3, 7). The following evidence suggests this mechanism: CYP7A1 enzyme activity is strikingly reduced hence the decreased bile acid synthesis when rats are fed with bile acids. In contrast, bile acid binding resin-caused interruption of bile acid reabsorption and reduction of enterohepatic circulation stimulates bile acid synthesis significantly. In addition to the negative mechanism, nutrients make contributions to bile acid synthesis as well. As CYP7A1 utilizes cholesterol as the substrate to synthesize bile acids, cholesterol *de novo* synthesis has an impact on bile acid synthesis. Evidence also suggests that insulin and glucose induce bile acid synthesis rapidly by activating *Cyp7a1* transcription (8). The regulation mechanisms maintain bile acid homeostasis and prevent hepatocytes from cytotoxicity caused by excessive bile acid accumulation. Imbalance of bile acid homeostasis will lead to various diseases. Therefore, serum bile acid levels are used as a biomarker for certain diseases.

Traditionally, bile acids serve important biological functions. As bile acids are synthetized from cholesterol, it is the major route to prevent cholesterol accumulation in human body. Therefore, they are quite important for cholesterol homeostasis. Due to the facial amphipathicity, especially when they are conjugated by polar amino acids, bile acids can form micelles with phospholipids and help solubilizing cholesterol to avoid precipitation of cholesterol in the gallbladder. Similarly, such property makes bile acids good surfactants and detergents to stimulate solubilization and absorption of cholesterol, lipids, and vitamins in the

intestine when the bile is secreted into the intestine lumen.

In addition to its role in lipid digestion and cholesterol homeostasis, over the past decades, bile acids have been discovered to be regulatory and signaling molecules like hormones by binding and activating a wide range of receptors (including nuclear receptors and G protein coupled receptors) and some cell signaling pathways like JNK1/2, AKT, and ERK1/2. Alterations of signaling pathways then further change many downstream genes. These genes are widely involved in the regulation of bile acids, lipid, and energy metabolism. Herein we will discuss the important receptors and pathways involved with bile acid signaling.

1.1.2 Signaling of bile acid-activated nuclear receptors

Nuclear receptors refer to a kind of transcription factors activated by ligands, which play vital roles in biological development and metabolism (9, 10). The general structure of nuclear receptors is composed of DNA-binding domain at the N-terminal and ligand-binding domain at the C-terminal. DNA-binding domain contains Zinc finger motifs and binds to a consensus AGGTCA repeating DNA sequence (direct, averted, or inverted repeat). Binding of ligands like bile acid molecules to the receptors via a LXXL motif at the ligand-binding domain enables the nuclear receptors to interact with the coactivators and change the conformation of the receptors, replacing the initial corepressors. As a result, the assembly of transcriptional complex is enhanced (as shown in Figure 3). Bile acids are found to activate three kinds of nuclear receptors: farnesoid X receptor (FXR) (11), pregnane X receptor (PXR) (12), and vitamin D receptor (VDR) (13). These receptors are widely expressed in many tissues, especially those exposed to bile acids such as the liver and the intestine (14, 15).

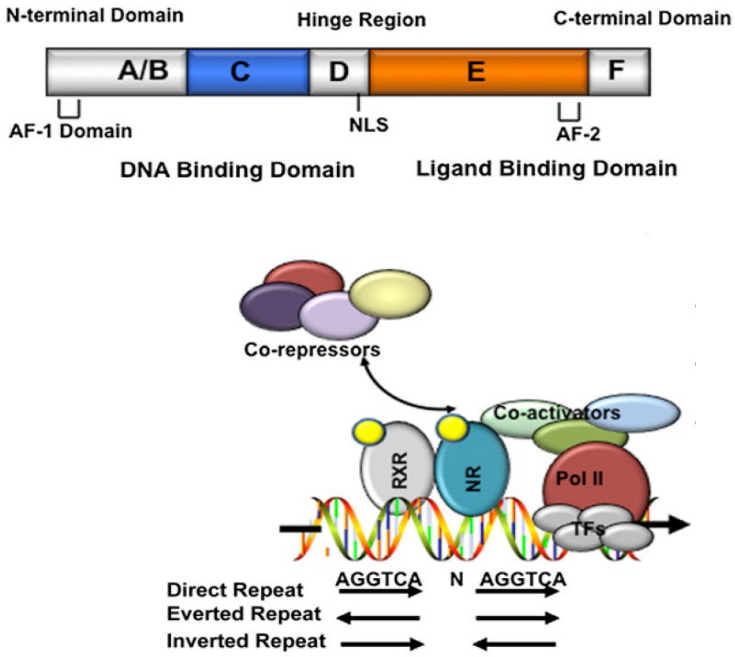

Figure 3 Structure and mechanism of action of nuclear receptors

Typical domains of nuclear receptors are shown on the top and the mechanism of action is presented below. Nuclear receptor response element binding sequences could be in direct, everted, or inverted repeat. Once activated by ligands, the corepressors will be replaced with newly recruited co-activators to activate the transcription of the target genes. AF-1, activation function 1; AF-2, activation function 2. NLS, nuclear localization sequence. Reproduced from "*Bile Acid Signaling in Metabolic Disease and Drug Therapy*" (6) with permission of American Society for Pharmacology and Experimental Therapeutics (ASPET).

FXR can be activated by farnesol, which is the origin of its name. It forms heterodimers with retinoic acid receptor (RXR) to bind specific DNA sequences in promoters and regulate transcription of target genes. Several FXR-response elements have been described amongst which IR-1 (two inverted repeats of a sequence AGGTCA separated by one base) has the highest affinity (16). Both of unconjugated and conjugated bile acids can activate FXR and the potencies of activation FXR are CDCA, DCA, LCA and CA (in descending order). A lot of studies have unraveled the important physiological roles of FXR in regulation of bile acid

homeostasis, energy and glucose homeostasis, and anti-inflammatory responses.

As mentioned above, bile acid synthesis can be regulated via a negative feedback mechanism. Actually, FXR is also found to participate in regulation of bile acid synthesis, secretion, and transport. *Cyp7a1* expression is higher in FXR knockout mice, leading enhanced biosynthesis of bile acids (17). The negative effect of FXR on bile acid synthesis is mediated via at least two mechanisms: SHP/LRH-1 pathway and FGF19/FGFR4 pathway. Liver-related homolog-1 (LRH-1) and hepatocyte nuclear factor (HNF) 4α can stimulate *Cyp7a1* gene transcription. When small heterodimer partner (SHP) is induced by FXR, SHP interacts with LRH-1 or HNF4α as a corepressor to inhibit their transactivation activities, leading to inhibition of CYP7A1 finally (18, 19). In addition, FXR synthetic agonist GW4064 induces fibroblast growth factor (FGF) 19 in hepatocytes (20). FGF19 then activates fibroblast growth factor receptor (FGFR) 4 in an autocrine or paracrine manner to inhibit CYP7A1 expression. While it was ever demonstrated that in human beings FGF19/FGFR4 mainly repress CYP7A1 expression by phosphorylating mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) signaling pathway (20), the downstream targets after MAPK/ERK signaling leading to inhibition of CYP7A1 are still largely unknown. FXR also regulates various transporters responsible for bile acid and cholesterol transport. In the liver, FXR inhibits transcription of Na+-dependent taurocholate cotransport peptide (NTCP) which uptakes bile acids into hepatocytes from portal blood (21). As contrast, FXR promotes expressions of the bile salt export pump (BSEP) secreting conjugated bile acids into bile (22), the multidrug resistant protein 2/3 (MDR2/3) exporting phosphatidylcholine into bile, the multidrug resistance-related protein 2 (MRP2) exporting organic anions (23), and ATP binding cassette G5/G8(ABCG5/G8) exporting cholesterol into bile (24). In the intestine, FXR activates organic solute transporter α and β (OSTα/β) in the basolateral membrane which excrete bile acids into portal circulation (25, 26) and reduces the expression of sodium-dependent bile

salt transporter (ASBT) in the apical membrane which absorbs conjugated bile acids into enterocytes (27).

The role of FXR in regulation of energy metabolism has long been perceived. Previous studies in mice found that FXR knockout mice accumulate more lipids in the liver and higher circulating levels of total cholesterol and triglycerides. Meanwhile administration of FXR agonist GW4076 or overexpression of FXR in the liver lowers circulating total cholesterol and triglycerides (28). Other studies reported that FXR represses induction of steroid response element binding protein (SREBP)-1c (29) and inhibits the transactivation of carbohydrate response element binding protein (ChREBP). As the result, hepatic lipogenesis and very low-density lipoprotein (VLDL) overproduction are reduced. In addition, FXR also induces apolipoprotein C-II (ApoC-II) and ApoA-V that activate lipoprotein lipase involved in triglyceride lipolysis (30, 31), resulting in facilitating triglyceride clearance in the end.

Some studies linked FXR to hepatic glucose metabolism, finding that FXR modulates circulating glucose level and regulates insulin resistance. An earlier study showed that activation of FXR inhibits two gluconeogenic genes: phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in the liver in a SHP-dependent way (32). This result is also consistent in mouse models of diabetes (28). In addition, it has been shown that insulin/AKT pathway (33) is stimulated upon FXR activation in the liver, causing enhanced glycogenesis. In pancreatic beta cells, FXR upregulates glucose-induced insulin transcription and secretion (34). Conversely, FXR-deficiency was found to elevate serum glucose and to impair insulin and glucose tolerance in mice (35). In obese mice, further activation of FXR with GW4064 improves insulin sensitivity (36). Therefore, FXR may impair gluconeogenesis but increase glycogenesis at the same time so as to improve glucose tolerance and insulin sensitivity.

Previous studies showed that FXR helps to antagonize inflammation during various liver injuries (37, 38), probably by inhibiting antagonizing nuclear factor-κB (NF-kB) to reduce proinflammatory cytokine production. Proinflammatory cytokines such as TNFα and IL-1β could inhibit bile acid synthesis the other way around. The details about the role of FXR in inflammation are still not so clear and need continuous study in future.

The other two nuclear receptors activated by bile acids: PXR and VDR, also play important roles. The xenobiotic receptor PXR represses hepatic CYP71A activity and mRNA expression (39, 40). Further studies indicated that repression of CYP7A1 by PXR is via inhibition of the transactivation activity of HNF4α and peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1a) on CYP7A1 (41, 42). Overexpression of PXR specifically in the liver induces hepatic lipid accumulation (43). VDR is also shown to repress CYP7A1 mRNA expression in human primary hepatocytes (44).

To make a short summary, activation of nuclear receptors by bile acids triggers multiple and ample intracellular signaling pathways. These receptors form a complicated regulatory network and coordinate various metabolic activities.

1.1.3 Signaling of bile acid-activated G protein coupled receptors

In addition to genomic actions of bile acids mediated by nuclear receptor, the non-genomic functions of bile acids were not established until G protein-coupled receptors (GPCRs) activated by bile acids were characterized. GPCRs are typically associated with guanine nucleotide-binding proteins (G proteins), contain seven transmembrane-spanning segments, and form the largest protein super family with over 800 receptors in mammalians (45). Currently there are at least four GPCRs modulated by bile acids: muscarinic receptors, formyl-peptide receptors (FPRs), TGR5, and sphingosine-1-phosphate receptor 2 (S1PR2). Bile acids were reported

to regulate activities of different muscarinic receptors (46). Impairment of muscarinic receptors may lead to some metabolic diseases (47). FPRs are thought to be inhibited by bile acids and to affect bile acids' anti-inflammatory properties (48, 49). For muscarinic receptors and FPRs, the physiological significance of bile acids' modulation has not been analyzed and studied in depth.

TGR5 is the GPCR that has been most studied in terms of bile acid signaling since it was first discovered in 2002 (50). TGR5 is a class A GPCR associated with G_s-protein, which mediates cAMP accumulation and triggers a variety of intracellular physiological activities. It is detected in different organs and tissues with different extents of expression. Gallbladder epithelium and the intestine express most TGR5 (50, 51). Similar to FXR, TGR5 also contributes to regulation of bile acid metabolism, energy and glucose homeostasis, and immune response.

Tgr5$^{-/-}$ mice have decreased total bile acid pool size in contrast to wild-type mice (52). The underlying mechanism, however, is not very clear as the hepatic expression of CYP7A1 is not changed and fecal bile acid secretion is normal in *Tgr5*$^{-/-}$ mice. Studies in *Tgr5*$^{-/-}$ mice in recent years demonstrated that deficiency of TGR5 leads to increased hydrophobic bile acid level and more severe liver injury under circumstances of overloading of bile acids like bile duct ligation or bile acid feeding (53). Taken together, the observations from TGR5 null mice suggest a role of TGR5 in bile acid homeostasis, but the related mechanisms are still largely unknown.

TGR5 is found to modulate energy homeostasis. Watanabe *et al.* showed that a lithogenic diet reduces high-fat diet induced body weight gain (54). Such a phenomenon should be ascribed to elevated energy expenditure instead of reduced intake. TGR5-cAMP mediated pathway induces deiodinase 2 (D2) and converse thyroxine (T4) to the active 3,5,3'-tri-iodothyronine (T3) in brown adipose tissue and muscles, which activates thyroid hormone receptor furthermore and consequently

increases mitochondrial oxidative phosphorylation and energy expenditure (54). The effect of bile acids on energy homeostasis is unrelated to FXR as FXR agonist fails to avoid diet-induced obesity and cannot reduce energy expenditure. In line with the above results, *Tgr5*$^{-/-}$ mice have increased body weight or a tendency toward higher fat content (52). Despite conclusions from mouse models, a recent clinical study did not find significant correlations between bile acid levels and energy expenditure (55). Thus, more evidence about the role of TGR5 with respect to energy expenditure in humans is still needed in future.

Previous studies have linked bile acids to glucose homeostasis via activation of TGR5 (56, 57). Glucagon-like peptide-1 (GLP-1), the peptide known to promote insulin secretion, is stimulated by bile acid-activated TGR5 in mouse enteroendocrine STC-1 cells (58). Treatment with a specific TGR5 agonist, 6α-ethyl-23(S)methyl-cholic acid (6EMCA or INT-777) raises ATP/ADP ratio and elevates GLP-1 secretion as well (59). While the TGR5 gain-of-function in mouse models produces relatively consistent and reproducible results, there are seemingly inconsistent and gender differences in results from *Tgr5*$^{-/-}$ mice. For example, the high fat diet displays impaired insulin sensitivity in male *Tgr5*$^{-/-}$ mice but improved insulin sensitivity in female *Tgr5*$^{-/-}$ mice (60). Similarly, another study found that only female instead of male *Tgr5*$^{-/-}$ mice gain higher body weight (52). Since TLCA and LCA are two of the most potent bile acids that activate TGR5 and the intestine is mostly posed to LCA, one may infer that there is no sufficient bile acid in the circulating system to activate TGR5 in addition to the liver and the intestine, hence the insignificant effect of disruption of TGR5 on the gain of body weight. Detailed mechanisms still need be studied in depth.

As TGR5 is highly expressed in spleen and immune cells like monocytes and macrophages (61), it is not strange that TGR5 is found to be related to an anti-inflammatory role in different metabolic and inflammation related diseases (61, 62).

Transfection of TGR5 into human THP-1 cells decreases LPS-induced TNF-α expression (61). In addition, treatment of rat Kupffer cells with a TGR5 agonist causes lowered expression of LPS-induced IL-1, IL-6 and TNF-α (63). The anti-inflammatory role of TGR5 in the liver has also been descripted (64), probably due to activation of TGR5 in Kupffer cells as TGR5 is not expressed in hepatocytes.

The last reported GPCR activated by bile acids is S1PR2. S1PR2 is a recently characterized GPCR activated only by conjugated bile acids (65). The natural ligand of S1PR2 is sphigosine-1-phosphate (S1P), a phosphorylated product from sphingosine by sphingosine kinase 1 (SphK1) and SphK2. S1P acts as a bioactive mediator and activates five specific cell surface GPCRs: S1P receptor 1-5 (S1PR1-5) (66). Conjugated bile acids like TCA was shown to activate S1PR2 in rat hepatocytes and subsequently activate ERK and Akt signaling pathways. Structural modeling shows that TCA can only dock into S1P binding site in S1PR2 instead of the other S1PRs (65).

S1P/Conjugated bile acids-activated S1PR2 signaling pathways are engaged in lipid and glucose metabolism and contribute to bile duct cancer, as shown in Figure 4. A previous study has shown that S1P is able to inhibit histone deacetylase 1 and 2 (HDAC1 and HDAC2) involved in epigenetic regulation (67). In S1PR2-deficient mice, genes related to lipid metabolism like SREBP-1c, LDLR, PPARγ, and FXR are substantially down regulated in the liver (68). Another study based on primary rat hepatocytes showed that TCA rapidly inhibits transcription of PEPCK and G-6-Pase but induces transcription of SHP. In addition, glycogen synthase kinase 3 (GSK3) is also phosphorylated and inhibited by AKT in rat primary hepatocytes. Therefore, the glycogen synthesis is enhanced, demonstrating insulin-like activity upon bile acids activation (69). A recent study found that activation of S1PR2 by conjugated bile acids promotes invasive growth of Human HuCCT1 cholangiocarcinoma cell line and upregulates the expression of cyclooxygenase-2(COX-1), making S1PR2 a

novel therapeutic target for cholangiocarcinoma (70).

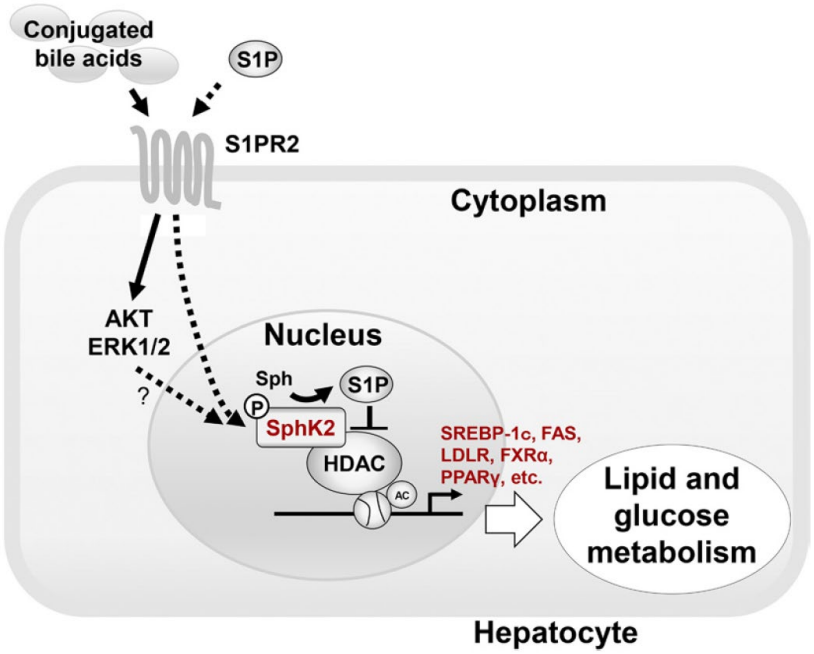

Figure 4 Regulation of bile acids and S1P on nutrient metabolism in the liver

Conjugated bile acids and S1P activate Sphk2 through S1PR2-activated AKT or ERK1/2 signaling pathways, hence the elevated nuclear S1P levels. Histone deacetylases (HDACs) are then inhibited by S1P and increase the acetylation of histones. Target genes involved in lipid and glucose metabolism are further upregulated.

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1.2. Adrenal gland: structure, function and regulation

1.2.1 Structure and function of mammal adrenal glands

Adrenal glands are a pair of triangular-shaped organs located on the top of kidneys. Each adrenal is divided into two basic parts: external adrenal cortex and internal adrenal medulla.

Adrenal medulla secretes hormones to help human beings deal with physical and emotional stress. Epinephrine (adrenaline) and norepinephrine (noradrenaline) are the two hormones secreted by the adrenal medulla. In order to respond to stress, these hormones work together to increase heart rate and rush more blood into the brain and muscles.

Adrenal cortex, however, plays a quite important and vital role for life. It consists of different zones (from the outmost to the innermost): *zona glomerulosa*, *zona fasciculata*, and *zona reticularis*. *Zona glomerulosa* is composed of columnar cells and produces the mineralocorticoids, mainly aldosterone. Aldosterone is mediated by signals from the kidney and affects mineral homeostasis such as facilitating potassium excretion and increasing sodium reabsorption in the kidney. Therefore, aldosterone maintains the balance between water and salt. *Zona fasciculata* makes up over 70 percent of the adrenal cortex and consists of polyhedral and columnar cells, which produce glucocorticoids. Glucocorticoids mainly include three hormones: cortisol (hydrocortisone), corticosterone and cortisone and they mainly influence glucose, protein and lipid metabolism. Cortisol is the most abundant of the three glucocorticoids in humans. Glucocorticoids act via a nuclear receptor: glucocorticoid receptor (GR) through transcriptional regulation of target genes. The acute glucocorticoid secretion will mobilize amino acids and fatty acids, stimulate glucose synthesis in the liver, and enhance the lipolysis in adipose tissues. Therefore, glucocorticoids have anti-insulin effect. In addition, glucocorticoids have anti-inflammatory and suppress "inflammation reaction". They are also immunosuppressive, which is quite obvious when they are administered at pharmacologic doses (72, 73). *Zona reticularis* is the innermost zone and made up of polyhedral cells while arranged as round nests. This zone produces gonad corticoids such as androgens and estrogens, which maintain the secondary sexual characteristics.

Mouse adrenal glands, however, are slightly different from human adrenals in that rodent adrenal gland does not have a distinguishable *zona reticularis* from *zona fasciculata* as 17α-hydroxylase is lacked (74). Therefore, corticosterone instead of cortisol is the dominant glucocorticoid in rodents. In addition, mouse adrenal cortex has a special and unique X-zone of which the function is still unclear, but it is perceived to be a post-partal remnant of the fetal adrenal zone (75) and only exists within a specific period (degenerates after puberty for males and after the first pregnancy for females).

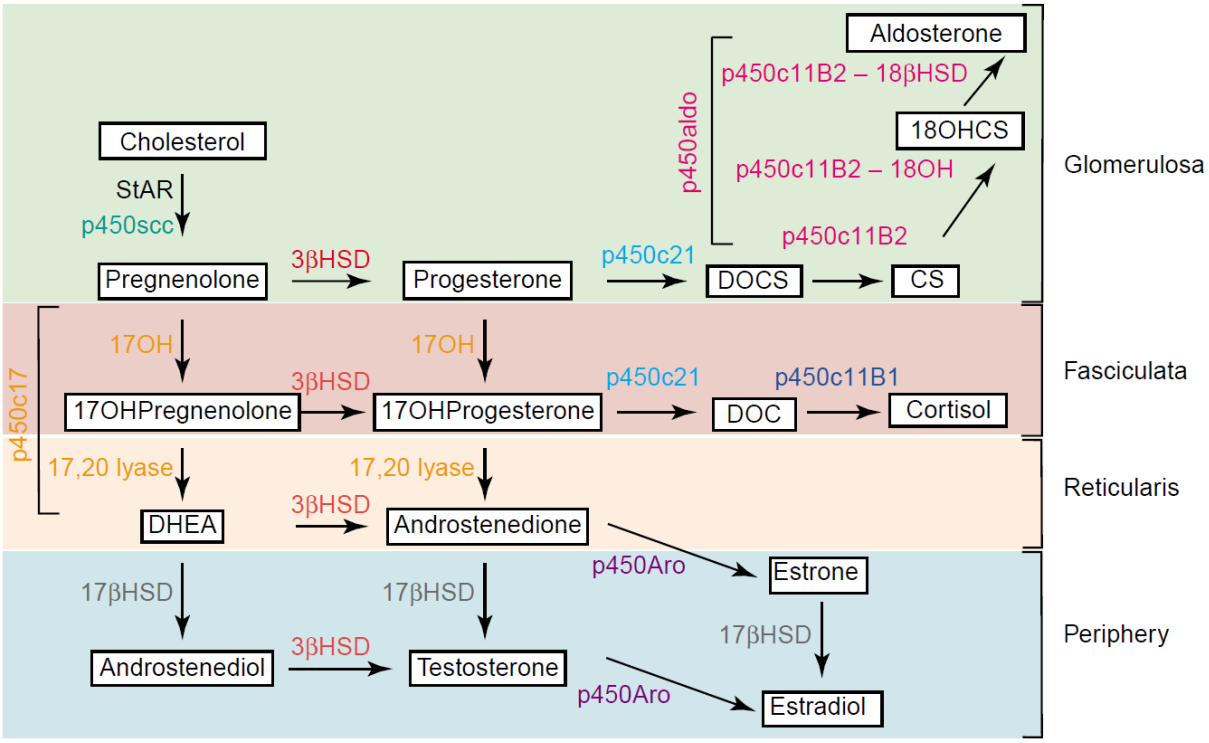

Figure 5 Steroidogenesis pathways in the human adult adrenal cortex

Glucocorticoid, mineralocorticoid, and androgen biosynthetic pathways are indicated in different zones of the cortex. Abbreviations for enzymes are listed as follows: StAR, steroidogenic acute regulatory protein; p450scc, side-chain cleavage enzyme; 3βHSD, 3β-hydroxysteroid dehydrogenase; p450c21, 21-hydroxylase; DOCS, deoxycorticosterone; p450aldo, aldosterone synthase, containing 11β-hydroxylase (p450c11B2), 18-hydroxylase (18OH) and 18β-hydroxysteroid dehydrogenase (18βHSD) activities; CS, corticosterone; p450c11B1, 11β1-hydroxylase; 18OHCS, 18-hydroxylase corticosterone; DOC deoxycortisol; p450c17, enzyme complex containing 17-hydroxylase (17OH) and 17,20 lyase activities; p450Aro, aromatase; DHEA, dehydroepiandrosterone;17βHSD, 17β-hydroxysteroid dehydrogenase.

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1.2.2 Metabolism of glucocorticoids

The synthesis process of glucocorticoids shares some common steps and substrates with the other steroids like mineralocorticoids and gonad corticoids. Briefly, steroidogenesis is initiated by steroidogenic acute regulatory (STAR) protein in the mitochondria which helps cholesterol move from the outer mitochondrial membrane into the inner mitochondrial membrane (76). The following stepwise conversions and modifications are mainly catalyzed by cytochrome P450s and hydroxysteroid dehydrogenases (See Figure 5.) in corresponding zones.

In humans, when cortisol is secreted by the adrenal gland, it will be converted to cortisone, the inert cortisol, by 11β-hydroxysteroid dehydrogenase (11β-HSD). There are two isoforms of 11β-HSD. 11β-HSD type 1 (11β-HSD1) convert cortisone to cortisol in humans, or 11-dehydrocorticorsterone (11-DHC) to corticosterone in rodents, while 11β-HSD type 2 (11β-HSD2) catalyzes the reaction in a reverse direction. There are some differences in terms of tissue distributions of the two isoforms. 11β-HSD1 is expressed in the liver, adipose tissue, and kidney. In contrast, 11β-HSD2 is mainly expressed in the kidney and salivary gland (77). Over 95% of the circulating cortisol is bound with albumin or corticosteroid-binding globulin (CBG) to inactivate cortisol (78). Therefore, the CBG levels in blood and tissues influence cortisol's bioavailability and clearance. Cortisol and cortisone are cleared metabolically in the liver initiated by A-ring reductases. Cortisol is finally metabolized into 5α and 5β tetrahydrocortisol (THF) and cortisone is converted into 5β-tetrahydrocortisone (THE) in the end. These metabolites are eliminated by the kidney in the form of urine (79) (Figure 6).

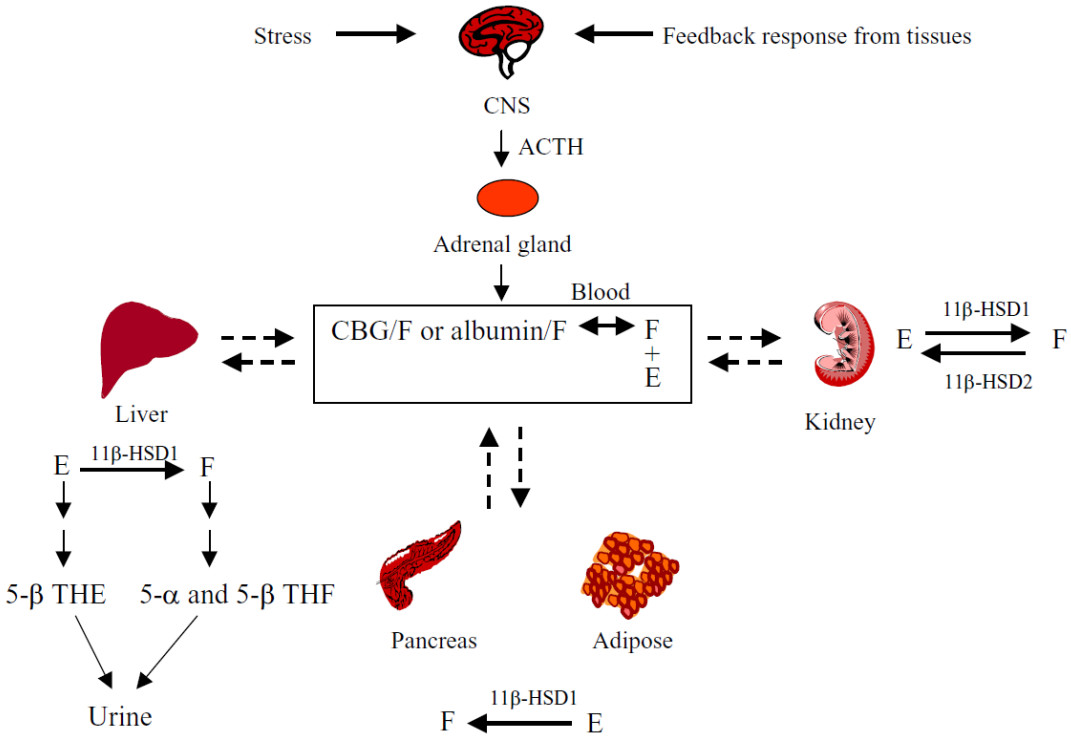

Figure 6 Schematic of glucocorticoid metabolism.

Glucocorticoid synthesis is regulated by the HPA axis. Plasma cortisol (F) is protein bound with 4%-5% free fraction and the plasma cortisone (E) is the free unbound form. Equilibrium of cortisol and cortisone is illustrated with dotted and bidirectional arrows. Glucocorticoids are metabolized into tetrahydrocortisone (THE) and tetrahydrocortisol (THF) in the liver and finally excreted into the urine.

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1.2.3 Regulation of hypothalamic-pituitary-adrenal (HPA) axis on glucocorticoids

Hypothalamic-pituitary-adrenal (HPA) axis is a complex and intact neuroendocrine system to maintain internal environment at a stable state when bodies are faced up with various stress, thus play a great role in the regulation of glucocorticoids under

physiological conditions.

Hypothalamus is located at the top most level in the HPA axis, receiving different signals from the brain. External signals such as stress and illness will make hypothalamus secrete corticotropin-releasing hormone (CRH), which is a 41 amino acid-long peptide. CRH activate the transcription of pro-opiomelanocortin (POMC) in the anterior pituitary. POMC is then further cleaved into multiple peptides including adrenocorticotropic hormone (ACTH), which stimulate glucocorticoid synthesis in the adrenal gland. Glucocorticoids in turn act at extrahypothalamic centers, the hypothalamus, and the pituitary gland to inhibit the stress response, thereby establishing a feedback loop (81-83).

When ACTH reaches the adrenal glands, it binds to the melanocortin type 2 receptor (MC2R) on cells of *zona fasciculata*. As a small G-protein-coupled receptor, MC2R contains a 7-transmembrane domain and belongs to a family of melanocortin receptors. Once bound by ACTH, it activates G_s protein and then stimulates adenylate cyclase, leading to elevated intracellular cAMP level, enhanced protein kinase A (PKA) activity and stimulated MAPK-dependent signaling cascades (84, 85). Some nuclear transcription factors such as cAMP response element (CRE) binding and CRE modulator (CREM) will also be influenced by PKA and further regulate a wide range of genes related to cholesterol metabolism and glucocorticoid synthesis. Specifically, it has been shown that ACTH stimulation enhances the transfer of cholesterol via high-density lipoprotein (HDL), as well as promotes delivery of cholesterol into mitochondria (86). Cholesterol biosynthesis is increased in response to ACTH treatment (87). The activity of hormone-sensitive lipase (HSL), a substrate of PKA, is elevated by ACTH treatment in rat adrenal primary cells (85). RNAs specific to steroidogenic enzymes like P450scc, CYP21, and CYP11B and CYP17A1 were also increased upon ACTH treatment (88-92). Notably, some steroidogenic enzymes like STAR, are not only regulated at transcription level, but

also influenced upon PKA phosphorylation as PKA substrate (93). Therefore, the effect of HPA axis on adrenals is hierarchical, multi-dimensional, and complex.

1.3 Bile acids and adrenal glands

1.3.1 Adrenal gland disorders and liver diseases

Adrenal gland disorders refer to situations when the adrenal glands cannot work properly as usual. Under such circumstances, there could be some problems with the adrenal gland itself, or disorders from other glands such as the pituitary. As a result, the adrenal gland produces too much or too little hormone, giving rise to serious health problems.

Cushing's syndrome and Addison's disease are two representative adrenal gland disorders. Cushing's syndrome, or hypercortisolism, is an adrenal gland disorder characterized by excessive circulating cortisol level and typically resulting from higher ACTH secretion due to adrenal adenoma or pituitary adenoma. In contrast, Addison's disease, or adrenal insufficiency (AI), refers to the adrenal disorder when the adrenals fail to produce sufficient cortisol. Primary adrenal insufficiency is due to destruction of the adrenal cortex mainly caused by autoimmune disorders or chronic infections (94). Second adrenal insufficiency, however, results from processes involving the pituitary gland and interfering with the production of ACTH. Tertiary adrenal insufficiency, similarly, involves the hypothalamus and due to lack of CRH or arginine vasopressin. Most common clinical manifestations in adrenal insufficiency include fatigue, lack of strength, anorexia, and salt craving. (95).

Most critically ill patients have appropriately elevated cortisol levels in relation to the severity of the stress, originally ascribed to the upregulation of the HPA axis (96). ACTH levels in these patients are, however, inappropriately low in comparison to the elevated cortisol levels for this explanation, leading to the term "ACTH-cortisol-dissociation" (97). In the setting of critical illness, the term "relative adrenal

insufficiency" (RAI) was first introduced in 1990s to describe the observation that there was an inadequate increment of serum cortisol after cosyntropin stimulation despite the basal cortisol level in patients with critical illness (98). Later on, the concept of "critical illness-related corticosteroid insufficiency" (CIRCI) was proposed in 2008 with a definition of "dysfunction of the HPA axis that occurs during critical illness" to replace the concept of RAI (99). Levels of cortisol in this condition are much higher than in healthy individuals, but are not sufficient to deal with the level of stress (100).

In liver diseases, the term "hepato-adrenal syndrome" is used to describe RAI in the setting of advanced liver disease and justified by its high occurrence in liver disease patients especially in patients with liver failure or post liver transplantation (101). However, due to lack of consensus on clinical diagnostic criteria as well as lack of understanding of the pathophysiological mechanisms, the range of reported prevalence of RAI in liver diseases is quite wide in literature. The prevalence of AI or RAI ranges between 7%-83% in patients with stable cirrhosis, and 10%-87% in critically ill cirrhotic patients, and 61%-92% in patients with liver transplant (102), which suggests a high prevalence of AI in both critically and non-critically ill cirrhotic patients. Conversely, some adrenal disorders also influence certain liver diseases. Some clinical studies in infants also indicated cortisol deficiency might contribute to pathogenesis of cholestasis in young infants (103-105). Addisons' disease, is also known to induce chronic liver damage (106).

1.3.2 Molecular links between bile acids and adrenal function

Considerable clinical and experimental evidence indicate that bile acid may play a regulatory role in the function of adrenal glands. First, jaundice was observed decades ago to be engaged to stop the progression of rheumatoid arthritis, which later enlightened the discovery of cortisol (107). In addition, impaired wound-healing together with higher incidence rates of mortality and sepsis were observed in

patients with jaundice caused by cholestasis who receive surgery and speculated to be connected with hypercortisolism (108, 109). More importantly, in contrast to those without cholestasis, serum cortisol concentrations in cholestatic patients were typically elevated (110). A previous clinical study (111) as well as our own unpublished pilot study also showed that levels of total bile acids were positively correlated with circulating total cortisol levels.

Previous studies have unraveled some molecular mechanisms of the regulatory role of bile acids on changes of glucocorticoid concentrations in blood. Expressions of 5-beta reductase, 11-beta hydroxysteroid dehydrogenase I and II in rat liver and kidney were inhibited by bile acids, leading to reduced breakdown of glucocorticoids (112, 113). In cholestatic rats, stress responsiveness of HPA axis was found to be suppressed (114) and CRH-mediated response was weakened substantially (115). Interestingly, bile acids were proven to enter the brain via ASBT. As a result, the expression of CRH was significantly inhibited (116), which could partially explain the observed HPA axis suppression in cholestatic rats.

The above studies showed that regulation of bile acids on glucocorticoid may happen either in the liver via influence on metabolism of glucocorticoids, or in the brain via interaction with secretion and release of CRH from hypothalamus. However, the specific and direct role of bile acids in regulating steroidogenesis in the adrenal cortex remains enigmatic but appears probable. Bile acids and glucocorticoids share similar molecular pathways in synthesis and catabolism. Importantly, some bile acids receptors such as FXR and TGR5 were detected in adrenal cortex cells (61, 117, 118)

2. Aims and approaches

In this thesis, whether adrenal function especially steroidogenesis pathway in the adrenal gland is influenced directly by bile acids at supraphysiological concentrations is studied. We used mouse cholestatic models as well as a human adrenocortical cell line H295R. Specifically, we performed common bile duct ligation (CBDL) in wild type and FXR knockout mice to model cholestasis with systemic retention of bile acids and cholephils. To study the sole effects of elevated serum bile acid levels *in vivo*, mice were also fed with a 1% CDCA supplemented diet. We cultured the human adrenocortical carcinoma cell line H295R for treatments with various bile acids as well as agonists or antagonists of potentially engaged pathways. Silent treatment with specific siRNA was also conducted in cell culture assays. Serum corticosterone or cortisol secretion levels, mRNA and protein levels of steroidogenesis-related genes, intracellular cAMP, and phosphorylation of target proteins in adrenals and cells were determined.

3. Materials and methods

3.1 Consumables, chemicals and reagents

Scientific research and experiments related to this thesis were mainly finished in Center for Medical Research (ZMF), Medical University of Graz. 1.5 mL tubes, pipettes and tips were purchased from Eppendorf, Germany. PCR-softstrips were purchased from Biozym Scientific, Germany. 15-50 mL centrifuge tubes, cell culture cryotubes, flasks with vented caps, 24-well plates were obtained from Corning, USA. Surgical instruments (forceps, tweezers, blades, syringes, etc.) and paraffin cassettes were bought from VWR international, Austria.

All bile salts were purchased from Sima-Aldrich, USA. JTE-013 and U0126 were obtained from Cayman, USA (Product No. Cay10009458 and Cay70970 respectively). Rp-isomer was obtained from Enzo Life, Switzerland (Product No. ALX-480-085). AC45594 was obtained from Tocris, UK (Product No. 3043). MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, was obtained from Sigma-Aldrich, USA (Product No. M5655). TRIzol® reagent, and Superscript II transcriptase were obtained from Invitrogen, USA. SYBR® Green PCR Master Mix, random hexamers, RNase inhibitor, were purchased from Applied Biosystems, USA. dNTPs was obtained from Applied Biosystems, USA. Oil red O Certistain® and Aquatex® were obtained from Merck, USA. Mayer's haematoxylin solution was obtained from Sima-Aldrich, USA.

3.2 Recipes for different buffer solutions

RIPA (Radioimmunoprecipitation assay) buffer contains 10 mM Tris-Cl (pH 8.0), 1% Nonidet P-40, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, and 150 mM NaCl. Oil red O stock solution contains 0.5 g oil red O Certistain® in 100 ml isopropanol. The powder is dissolved by using the gentle heat of a water bath at around 60°C. Buffers used in Western Blotting are as follows: running buffer

containing 25 mM Tris base, 190 mM glycine, and 0.1% SDS (pH=8.3); transfer buffer containing 25 mM Tris base, 190 mM glycine and 20% methanol (pH=8.3); TBST (Tris-buffered saline plus Tween 20) containing 10 mM Tris, 150 mM NaCl and 0.1% Tween-20 (adjust pH to 7.5); Blocking buffer containing 5% milk or BSA in TBST buffer, depending on the primary antibodies.

3.3 Methods

3.3.1 Animal experiments

All animal experiments were approved by the local authorities (approved animal applications BMWF-66.010/0012-II/3b/2014 and BMWFW-66.010/0129-WF/V/3b/2016). Housing, care and management of animals were in conformity with the criteria outlined in Austrian “Tierversuchsgesetz 2012”. FXR and TGR5 wild type and knockout C57BL/6 mice were obtained from the National Institutes of Health (NIH, Bethesda, MD) (17) and from the Institut de Génétique et Biologie Moléculaire et Cellulaire, Illkirch, France (59) respectively. Mice were housed at the Center for Medical Research in Graz with a 12 hours’ dark cycle (18:00-6:00) and 12 hours’ light (6:00-18:00) and had access to food and water *ab libitum*. Each cage contained up to four mice to avoid overcrowding. For common bile duct ligation (CBDL), 13-20 weeks old male wild type C57BL/6 mice and corresponding genotype knockout littermates were used. Mice were anaesthetized with isoflurane and placed in supine position. CBDL surgery was performed by firstly disinfection of mouse abdomen and transecting the integument from the urinary bladder to the sternum through the linea alba. Once abdominal cavity was opened, the common bile duct just hid under the liver lobes. Two separated sutures were used to tie up the common bile duct and cystic duct. Gallbladder was subsequently removed after bile duct ligation. After that, the abdominal cavity was stitched up carefully and mice were put back to cages until recovery. Sham operation followed similar protocol without the ligation of bile duct as described previously (119). Then mice continued to be housed for 7 days

(to model more acute effect of cholestasis) or three weeks (to model more chronic effects of cholestasis) before harvesting. For CDCA-feeding, one group of mice were fed with standard chow diet while another group of mice were fed with a 1% CDCA-supplemented diet for 5 days. Each group contained at least 3-6 mice in all mouse experiments.

In the end, all mice were sacrificed by cervical dislocation in the afternoon around 18:00. Trunk blood was obtained by decapitation for further blood analysis. Livers and adrenal glands were carefully removed from the abdominal cavity, immersed into pre-cooled methyl butane by dry ice, and snap-frozen in liquid nitrogen. Before cryopreservation, adrenals should be carefully trimmed to remove adherent fat tissues. An automated analyzer Hitachi 917 (Boehringer Mannheim, Germany) was used for biochemical measurements in mouse blood samples. To determine serum/plasma corticosterone levels, a mouse/rat corticosterone ELISA kit (Enzo Life, Switzerland) was used according to the official manual. To determine plasma ACTH levels, a mouse ACTH ELISA kit (Sigma-Aldrich, USA) was used according to the official manual.

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3.3.2 Lipid extraction and measurement in mouse adrenals

Mouse adrenals were picked up and snap-frozen. Each mouse adrenal were homogenized with 300 µL of PBS solution. 100 µL of homogenate was used for total protein measurement. Remaining 200 µL of homogenate was transferred in a glass vial. 1200 µL of mixture of chloroform and methanol (2:1, 800µL chloroform and 400µL methanol) was added and mixed thoroughly by vortex for 30 seconds. Then additional 100 µL of PBS was added and mixture was subjected to vortex for 15 seconds. After that, the mixture was centrifuged for 20 minutes at 8000 rpm to make lipid abstract enter the bottom phase. 200 µL of bottom phase was transferred into a new Eppendorf tube and dried in a vacuum centrifuge at 50°C. The dried pellet was dissolved with 10% Triton X-100 in 100% ethanol or directly dissolved in assay

buffer provided from Total Cholesterol Assay kit (Cell Biolabs, USA).

Cholesterol concentration was determined according to the manual. Briefly, each lipid sample was divided into two parts equally: one for free cholesterol measurement and the other for total cholesterol measurement. To determine free cholesterol, 50 µL of lipid sample together with cholesterol standards were added into a 96-well plate. Then 50 µL of the cholesterol reaction reagent was added and mixed well. After 45-minute incubation at 37°C, the plate was read by a spectrophotometer at 540-570 nm. Free cholesterol values in samples were calculated according to the standard curve. For total cholesterol measurements, cholesterol esterase needed to be added into the reaction reagent and the same steps were repeated to the other half part of each sample. Cholesterol ester concentrations were calculated by subtracting free cholesterol concentration from total cholesterol concentration. Final results were normalized to total protein of each adrenal sample.

3.3.3 Cell cultures

H295R cells used in this study were purchased from the American Type Culture Collection (ATCC CRL-2128). Culture medium was purchased from Thermo Fisher Scientific, USA (Catalog Number 31330038), which is a 1:1 mixture of Dulbecco's modified Eagle's medium with Ham's F-12 nutrient mixture with 15 mM HEPES buffer. 2.5% Nu-serum (Corning, USA) and 1% ITS premix universal culture supplement (Corning, USA) were added into DMEM/F-12 to reconstitute the complete growth medium. The final concentrations of additives of the complete growth medium are 6.25 µg/mL transferrin, 5.35 µg/mL linoleic acid, 6.25 ng/mL selenium, 6.25 µg/mL insulin, 1.25 mg/mL bovine serum albumin, and 2.5% Nu-serum. H295R cells were cultured in 75 cm^2 flasks with 12.5 mL of complete growth medium at 37°C with a 5% CO_2 atmosphere. The medium was changed two times a week. For subculturing, first cells were washed with sterile PBS to remove the

complete medium. Then 2.0 to 3.0 mL of trypsin-EDTA solution was added into the flask and the flask was laid in the incubator at 37°C until cell layer was dispersed (within 5 to 15 minutes). After cells were fully detached and dispersed, 6.0 to 8.0 mL of complete growth medium was added into the flask and cells were aspirated by gently pipetting. The cell suspension mixture was further transferred into a centrifuge tube and spun at 1000 rpm for 5 minutes. Supernatant was discarded and cell pellets were resuspended with appropriate complete growth medium at a subculture ratio 1: 4. For cryopreservation, first prepare freeze medium by mixing complete growth medium with extra 7.5% Nu-Serum (accounting for 95% of the freeze medium) and DMSO (accounting for 5% of the freeze medium). Confluent cells were treated as described in subculturing until cell pellets were harvested. Then freeze medium was used to carefully disperse cells and aliquots of cell suspension were transferred into cryotubes. Mr Frosty boxes were then used to cool down cryotubes gradually at -80°C overnight. Cryotubes were finally stored at liquid nitrogen vapor phase for long-term preservation.

For cell assays, H295 cells were seeded into 24-well plates. The complete growth medium was removed when H295R cells reached 100% confluent. Serum-free medium was added into each well and cells were incubated overnight. On the following day, 1 mL of fresh serum-free medium containing various concentrations of conjugated bile acids was added into each well, together with chemical inhibitors such as JTE-013 and U0126 where necessary. Cells were further incubated for another 24 or 48 hours before harvesting. The culture medium was collected in the end and H295R cells were broken open with RIPA buffer. Total protein levels were determined by the pierce BCA protein assay kit (Thermo Fisher, USA). Concentrations of cortisol secreted by H295R cells into the medium supernatant were measured with ADVIA Centaur XP Immunoassay System (Siemens, Germany) at the Endocrinology Lab Platform, Medical University of Graz. Cortisol levels were finally normalized to the total amount of protein in each well.

3.3.4 MTT assays

A stock solution of MTT was prepared at a concentration of 5 mg/mL by dissolving MTT powder in DMEM/F12 without phenol red (Product No. 21041 from Life Technologies, USA). After H295R cells were treated with bile acids for 48 hours, the culture medium was removed. Working solution of MTT was obtained by diluting stock solution at a ratio of 1:10 and added into each well. MTT solution was not aspirated until formazan crystals were converted after 3-4 hours. 400 µL of 0.1 N HCL (dissolved in isopropanol) was used to solubilize the purple crystals pro well and further subjected to photometric measurement. Absorbance values at the wavelength of 570 nm were recorded by the photometer, which are directly proportional to cell variability.

3.3.5 *Ex vivo* culture of mouse adrenals

Fresh medium was prepared with DEMEM/F12 medium from Life Technologies supplemented with 5% FBS and 1% antibiotics. Male mice were housed, euthanized, and killed as described in the section of " Animal experiments". Adrenal glands were picked up from mouse abdominal cavity. Adipose tissue surrounding adrenals was carefully excised. Each adrenal gland was bisected and soaked in fresh medium to flush away the adherent blood. After that, the washing medium was aspirated and 700 µL of fresh medium with or without specific concentrations of bile acids was added to completely cover all adrenal slices. Adrenal slices were then incubated for 2 hours at 37°C with a 5% CO_2 atmosphere. In the end, slices were washed with PBS and lysed with RIPA buffer.

3.3.6 Human materials

When brain dead and deceased organ donors were subjected to the explant surgery, their adrenal glands were located, excised from donors' body, and snap-frozen in liquid nitrogen as fast as possible for future protein analysis. The Institutional Review

Board of the Medical University of Graz approved the surgery (number of the ethic votum: EK-Number 21-176 ex 9/10).

3.3.7 Quantitative Real Time PCR (qRT-PCR)

Snap-frozen mouse adrenals were added to proper quantity of TRIzol® reagent and homogenized with plastic pestles. In contrast, H295R cells were lysed directly with addition of TRIzol® reagent. RNA extraction was performed as following steps:

- Soak mortals, pestles in acetone, and make them air dry on absorbing paper. Use UV crosslinker to sterilize pipettes.
- Add 600 µL of TRIzol® reagent into the 1.5-mL tube and put frozen adrenal glands in it. Crush, grind and homogenize the glands by pestle.
- Add 120 µL of chloroform and shake tubes for about 15 seconds, then subject tubes to tube rotators at 4°C for 20 minutes.
- Centrifuge the tubes at a speed of 13000 rpm for 15 minutes at 4°C. The liquid will be separated into three phases, and RNA is contained in the aqueous phase (upper phase)
- Transfer the supernatant into a new tube and add equal volume of precooled isopropanol, shake the tubes gently for a few seconds and incubate the tubes on ice for 5 minutes. Then centrifuge at 13000 rpm, for 20 to 30 minutes at 4°C
- Remove the supernatant by pipetting and add 500 µL of 70% precooled ethanol, vortex, and centrifuge at 13000 rpm for for 20 to 30 minutes at 4°C.
- Remove the supernatant by pipetting and air-dry the pellet for 5 to10 minutes.
- Add 15-20 µL of distilled, RNase-free water into the tube and incubate tubes at 55- 65°C centigrade for 10 minutes if necessary.

After determination of concentrations with Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), 1.5 µg of total RNA was used for reverse transcription. Total 20 µL of reverse transcription reaction mixture contains 2.7 µL of water, 4 µL of 5-fold reaction buffer, 2 µL of 0.1M DTT, 0.3 µL of dNTPs, 0.5 µL of

RNase inhibitor and 0.5 μL of Superscript II reverse transcriptase. The PCR program is: 65°C for 5minutes, 25°C for 10 minutes, 42°C for 90 minutes and 70°C for 15 minutes. cDNA samples were diluted 1:20 and transferred into new tubes to create cDNA pools. Synthetic cDNA samples were subjected to RT-PCR on a 384-well plate with 10 μL of mixture each well containing 5.25 μL SYBR® Green PCR Master Mix. Cycling program for real time PCR was set as: 95°C, 10 minutes; 40 cycles (plate was read at the end of each cycle) containing denaturation 95°C for 30 seconds, annealing 60°C for 1 minutes, extension 72°C for 30 seconds; 72°C for 10 minutes; 55°C to 95 °C , 0.2°C /read, 1 second hold for melting curve analysis. Specific primers used in qRT-PCR are listed in Table 1. Cyclophilin or 18S rRNA was used as the reference and housekeeping genes. Relative quantification of double standard curves was used to determine the relative concentration of cDNA copies in each reaction.

Table 1 Primer list

Gene	Accession Number	Primers
mouse_*Shp*	NM_011850	5'-AAGGGCACGATCCTCTTCAA-3'
		5'-GTACCAGGGCTCCAAGACT-3'
mouse_*cyclophilin*	NM_011149	5'-GGAGATGGCACAGGAGGAA-3'
		5'-GCCCGTAGTGCTTCAGCTT-3'
mouse_*Star*	NM_011485	5'-CCAGGAAGGCTGGAAGAAGG-3'
		5'-GTCTACCACCACCTCCAAGC-3'
mouse_*Hsd3b1*	NM_001304800	5'-TCCACACTGCTGCTGTCATT-3'
		5'-AGATGAAGGCTGGCACACTT-3'
mouse_*Cyp21a1*	NM_009995	5'-TCCAAGAGAGTCGGGACCAT-3'
		5'-CTTTCCATTGGCCTGCAACC-3'
mouse_*Cyp11b1*	NM_001033229	5'-CTGGGACGTGGTGTGTTCTT-3'
		5'-CCCTTGCTATCCCATCCACC-3'

mouse_*Abca1*	NM_013454	5'-CTCTTCATGACTCTAGCCTGGA-3'
		5'-ACACAGACAGGAAGACGAACAC-3'
mouse_*Sr-b1*	NM_016741	5'-GAGCACGTTCTACACGCAG-3'
		5'-GGTCTGACCAAGCTATCAGGTT-3'
mouse_*Hmgcred*	NM_001360165	5'-CCGGCAACAACAAGATCTGTG-3'
		5'-ATGTACAGGATGGCGATGCA-3'
mouse_*Cyp51*	NM_020010	5'-GTTGGGGAGAAAGCGGAGAA-3'
		5'-GAGCCACCTTCTCGTTGAGT-3'
mouse_*Mc2r*	NM_001301372	5'-ACACCGCAAGAAATAACTCCG-3'
		5'-AGGAGGACAATCAAGTTCTCCA-3'
human_*S1PR2*	NM_004230	5'-TCTCTACGCCAAGCATTATGTGC-3'
		5'-TGGCCAACAGGATGATGGA-3'
human_*STAR*	NM_000349	5'-TTGCTTTATGGGCTCAAGAATG-3'
		5'-GGAGACCCTCTGAGATTCTGCTT-3'
human_*HSD3B2*	NM_001166120	5'-GCGGCTAATGGGTGGAATCTA-3'
		5'-CCTCATTTATACTGGCAGAAAGGAAT-3'
human_*CYP21A2*	NM_001128590	5'-TCCCAGCACTCAACCAACCT-3'
		5'-CAGCTCAGAATTAAGCCTCAATCC-3'
human_*MC2R*	NM_001291911	5'-GACTGTCCTCGTGTGGTTTTG-3'
		5'-GGCTGCCCAGCATATCAGAT-3'
human_*FXR*	NM_001206979	5'-AGGGGTGTAAAGGTTTCTTCAGGA-3'
		5'-ACACTTTCTTCGCATGTACATATCCAT-3'
human_*TGR5*	NM_001077191	5'-GCTGCTTCTTCCTGAGCCTA-3'
		5'-GTTGGGAGCCAAGTAGACGA-3'
human_*18S RNA*	NR_003286	5'-CTCAACACGGGAAACCTCAC-3'
		5'-AGACAAATCGCTCCACCAAC-3'

3.3.8 Western Blotting

RIPA buffer was prepared as cell lysis buffer. Before immediate use, a thawed

aliquot of RIPA buffer was added with protease and phosphatase inhibitors. Mouse adrenals and H295R cells were collected, washed with PBS, and disrupted with a sonicator in RIPA buffer. The lysis solutions were then centrifuged at 9000 rpm for 20 minutes at 4°C. The supernatants were aspirated and stored at -20°C. A Pierce BCA Protein Assay Kit (Thermo Fisher, USA) was used to determine the total protein concentrations in homogenized cell lysates according to the official manual. For Western Blotting, 20-30 µg of total protein were mixed with 10-fold loading sample buffer and denaturized by incubation at 95°C for 5 minutes. Mixed protein samples were loaded into 10% SDS-PAGE gels together with molecular weight marker. The gel was run for 1.5 hours at 100 V to separate different sizes of proteins. Proteins in the gel were transferred to nitrocellulose membrane using transfer tank for 2 hours at 250 mA. The nitrocellulose membrane was blocked over night at 4°C in blocking buffer. Blocking buffer is TBST buffer (10 mM Tris, 150 mM NaCl and 0.1% Tween-20, pH=7.5) containing 5% milk or bovine serum albumin, depending on the primary antibodies. Primary antibody against target protein was added into the blocking buffer with appropriate dilutions and used to incubate the membrane overnight with agitation at 4°C. Antibodies used in this study are: STAR, CYP21, CYP11B and GAPDH, from Santa Cruz, USA; total ERK 1/2, phosphorylation of ERK1/2 at Thr202/Tyr204, from Cell Signaling, USA; β-Actin, from Sigma, USA; S1PR2 antibody used to detect protein expression in H295R cells, from Santa Cruz, USA; S1PR2 antibody used to detect protein expression in human adrenals, from Proteintech, USA. After incubation, the membranes were washed three times with TBST, 5 minutes each time. A peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Cell Signaling, USA) was applied. The membrane was incubated with secondary antibody with agitation for 1 hour at room temperature. The blot was then washed in TBST and PBS in a row. Finally, the blot was incubated with Western ECL Substrates (Bio Rad, USA). Chemiluminescent signals were detected using Bio Rad ChemiDoc™ Touch Imaging System.

3.3.9 Oil red O staining

Mouse adrenals were harvested and immediately snap-frozen in isopentane with dry ice. Frozen adrenals were then embedded in OCT compound. Cryostat sections were cut at 5-10 μm and mounted on gelatin-coated histological slides under a cryostat temperature between -15°C and -20°C. Tissues sections were air-dried at room temperature for 30 minutes. Dry sections were then fixed by immersion in cold acetone (-20°C) for 10 minutes. The working oil red O solution should be freshly prepared by mixing 3 parts of stock solution and 2 parts of distilled water prior to staining. For staining steps, the cryosections were first rinsed with 60% isopropanol for about 5 minutes. Then sections were stained with oil red O working solution for 15 minutes. The same 60% isopropanol was used to rinse the slides again. Dips of Mayer's haematoxylin solution were added to stain nuclei. After rinsed with distilled water, the cryosections were mounted with aqueous Aquatex® agents.

3.3.10 siRNA transfection

siRNA primers of S1PR2 were purchased from Dharmacon, USA (ON-TARGET plus SMART pool siRNA, Catalog number: J-003952), containing four pairs of primers. MISSION® siRNA universal control No.1 was obtained from Sigma-Aldrich, USA (Catalog number: SIC001). Lyophilized siRNA was resuspended with siRNA buffer to prepare a stock solution of 20 μM. Transfection assays were conducted according to the official protocol by Dharmacon with some modifications. Briefly, H295 cells were split and seeded at a density of 5×10^5 cells per well in 24-well plate. 5 μM S1PR2 siRNA and negative control were prepared in parallel beforehand. 2.5-μL 5 μM siRNA was diluted with serum free medium to a final volume of 50 μL. DharmaFECT transfection reagent No.1 was diluted by adding 2.5-μL reagent into 47.5-μL serum-free medium. 50-μL diluted siRNA and 50-μL diluted transfection

reagent were mixed gently and incubated for 20 minutes. Then 400-μL antibiotic-free complete medium was added into 100-μL mixtures of siRNA and transfection reagent. The final 1-ml solution was dispensed into each well. Final siRNA concentration for each well is 25 nM. Cells were incubated at 37°C with a 5% CO_2 atmosphere for 48 hours before the transfection medium was removed and replaced with complete growth medium with or without bile acids. Cells were cultured for another 48 hours before harvesting. Medium samples were also collected for further cortisol measurements.

3.3.11 Luciferase activity assay

H295R cells were cultured in 24-well plate as described above. Steroidogenic factor 1 (SF-1) luciferase reporter plasmid was a kind present from Prof. David D Moore in Baylor College of Medicine, Houston, Texas, USA (120). The promoter sequence on the plasmid before luciferase coding region contains 5 copies of SF-1-responsive elements. For transfection assays, H295R cells were co-transfected with 200 ng SF-1 luciferase reporter plasmid and β-galactosidase as internal control each well. As contrast, another group of cells were co-transfected with the same amount of pcDNA3 plasmid and β-galactosidase. Transfections were done in triplicates with lipofectamine 2000. Cells were incubated at 37°C with a 5% CO_2 atmosphere for 24 hours. Then the transfection medium was changed into complete growth medium with bile acids and cells were incubated for another 24 hours. To determine luciferase and β-galactosidase expressions, luciferase assay system and β-galactosidase assay system were purchased from Promega, USA. H295R cells were lysed with Reporter Lysis Buffer. A 96-well plate was placed in a LUMIstar Omega Luminometer. 20 μL of cell lysates was added into each well by the injector of the luminometer. Each well was then added 100 μL of Luciferase Assay Reagent

by the injector and read by the luminometer immediately. The inject-then-read process was repeated until all wells of the plate were injected and read. For β-galactosidase assays, 50 µL of the cell lysates was mixed with equivalent volume of Assay 2X Buffer and added into each well of the 96-well plate. The plate was incubated at 37°C for half an hour. 150µL of 1M sodium carbonate was then added to stop the reaction. Absorbance values at 420 nm were recorded by a photometer. Luciferase activity values were finally normalized using β-galactosidase activity as reflection of transfection efficiency.

3.3.12 Data analysis

GraphPad Prism 5.0 was used for preparations of graphic presentations and statistical analysis. Quantitation of protein bands was performed using Image Lab software from Bio Rad. Unpaired Student's t test was performed when the comparisons were made between two groups. One-way ANOVA was applied in multiple comparisons, which was followed by Holm-Sidak's multiple comparison test as *post hoc* analysis. *p*-values are two-tailed, and differences are considered significant with a *p*-value less than 0.05.

4. Results

4.1 Circulating corticosterone levels are strikingly increased in CBDL and CDCA-fed mice, which is FXR and TGR5-independent

CBDL in rodents as a model of obstructive cholestasis, has long been carried out in scientific research. It is characterized by systemic retention of cholephils including bile acids (121, 122). To study the influence of excessive accumulated circulating bile acids on adrenal functions, we first performed CBDL surgery in C57BL/6 mice. The serological measurements in the mice surely showed dramatically increased serum bile acid levels in CBDL mice which was more than 200-fold on average compared with the sham-operated mice. As a sensitive indicator of liver function, alanine transaminase concentrations in blood of CBDL mice were increased substantially as well in contrast to the control group, indicating server liver damages caused by CBDL surgery (Fig 7A). Corticosterone, the major glucocorticoid in rodents was chosen to evaluate adrenal function under such conditions. Interestingly, the serum corticosterone levels in CBDL mice were more than two times as high as those of the sham-operated mice (Figure 7 A). Meanwhile, the HPA axis activity was not significantly increased as the circulating ACTH level kept unchanged (Figure 7 A).

Although CBDL is a good model of obstructive cholestasis, bile acids are not the only cholephils or signaling molecules that may directly affect adrenal steroid synthesis and secretion. In addition, CBDL in mice leads only to a very small increase in serum levels of CDCA and its conjugate TCDCA which are the highly poten FXR agonists in contrast to CA as shown previously (123). In order to study the solo and first-hand effect of bile acids instead of any other cholephils on circulating levels of glucocorticoids, mice were fed a diet supplemented with 1% CDCA, the most abundant bile acid in humans, for 5 days. As shown in Figure 7 B, CDCA-supplemented diet also caused symptoms of cholestasis in in serological test

including elevated serum total bile acid and ALT levels. In addition, similar to CBDL mice, CDCA-fed mice did not change the ACTH levels in blood significantly but had doubled serum corticosterone concentrations in contrast to the chow control group.

FXR, as an important bile acid receptor, was previously reported to be expressed in adrenal cortex cells (15, 124). In addition, TGR5 mRNA has also been detected in adrenal glands (61). Therefore, both FXR and TGR5 knockout C57BL/6 mice were introduced for loss of function animal experiments. Interestingly, *Fxr*$^{-/-}$ mice also had higher levels of serum corticosterone, both following CBDL and CDCA feeding in contrast to sham-operated and chow-fed mice respectively (Figure 7 A and B). Cholestatic injury as determined by serum bile acid and ALT levels was higher in CBDL FXR knockout mice. This was paralleled by a more pronounced increase of corticosterone levels compared to their wild type littermates, indicating a correlation of adrenal dysfunction with the degree of cholestasis and, furthermore, strongly pointing towards an adrenal bile acid-dependent but adrenal FXR-independent mechanism. Likewise, serum corticosterone levels of CDCA-fed *Tgr5*$^{-/-}$ mice were still increased significantly in contrast to *Tgr5*$^{-/-}$ chow mice (Figure 8 A).

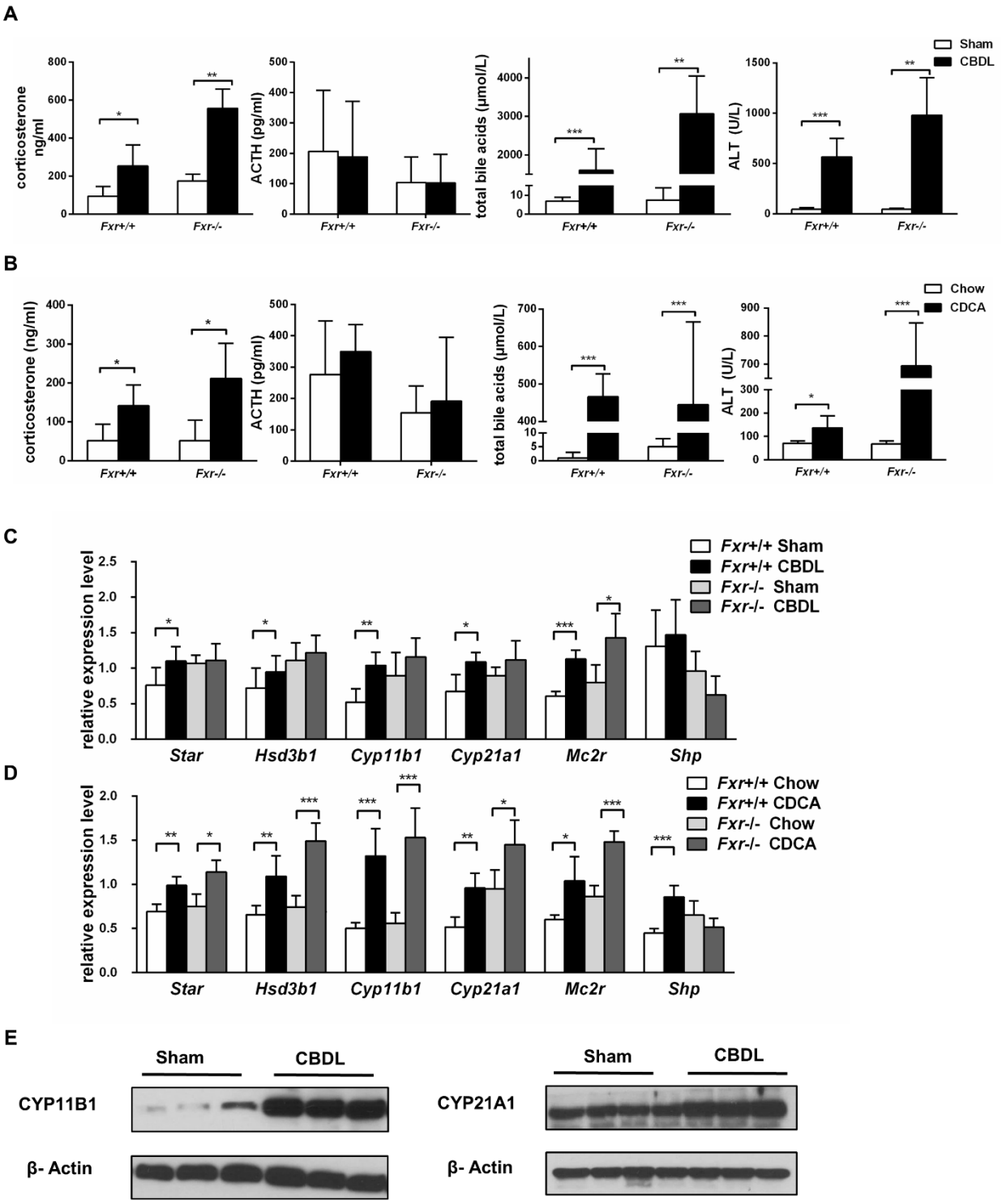

Figure 7 CBDL and CDCA-feeding boosted circulating corticosterone concentrations as well as steroidogenesis of adrenals in mice independent of FXR

Fxr$^{+/+}$ and *Fxr*$^{-/-}$ mice were subjected to CBDL for 7 days (**A, C**) or 3 weeks (**E**), or fed a diet supplemented with 1% CDCA for 5 days (**B, D**). Each group contained 3 to 5 mice. **A**, **B**:

Serological tests were performed with blood from CBDL or CDCA-fed mice. Total bile acid and ALT levels were abnormally high, showing symptoms of cholestasis and effectiveness of both treatments. Circulating corticosterone levels in both CBDL and CDCA-fed mice were significantly higher compared to the corresponding control group independent of FXR. There were no statistical differences with regard to plasma ACTH concentrations caused by CBDL or CDCA feeding. **C**, **D**: RNA samples were extracted from adrenal glands of 7-day CBDL and 5-day CDCA-fed mice for RT PCR analysis. All relative mRNA levels of genes were normalized to cyclophilin and presented as means ± standard deviations. Statistically significant changes are indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to sham or chow control group. Transcriptional expressions of *Star, Hsd3b1, Cyp11b1, Cyp21a1, Mc2r* were elevated in both CBDL and CDCA-fed mice. Changes of mRNA levels in CDCA-fed mice were independent of FXR. **E**: Adrenal gland from 3-week CBDL mice were subjected to Western Blotting assays. β-actin acted as the housekeeping protein. CYP11B1 and CYP21A1 were detected at a higher protein level in CBDL mouse adrenals. The figure is adapted from "*Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1"* (125) with permission of Wiley.

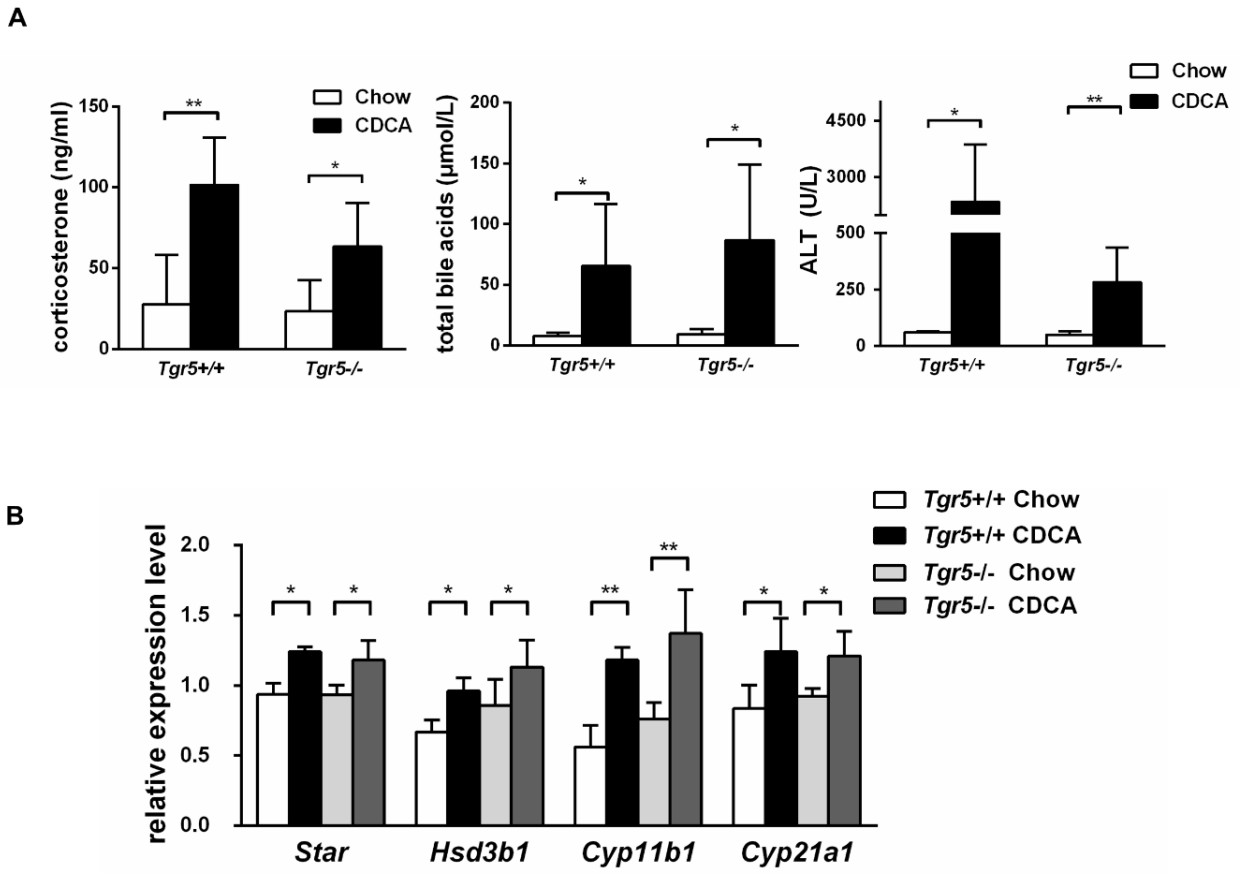

Figure 8 CDCA-feeding boosted circulating corticosterone concentrations as well as steroidogenesis of adrenals in mice independent of TGR5

Tgr5$^{+/+}$ and *Tgr5*$^{-/-}$ C57BL/6 mice were fed a diet supplemented with 1% CDCA for 5 days. Each group contained 3-5 mice. **A**: Serological measurements were performed from blood of CDCA-fed mice. Total bile acid and ALT levels were abnormally high, showing symptoms of cholestasis. Circulating corticosterone levels in CDCA-fed mice were significantly higher compared to the corresponding control group independent of TGR5. **B**: RNA samples were extracted from adrenal glands of 5-day CDCA-fed mice for RT PCR analysis. All relative mRNA levels of genes were normalized to cyclophilin and presented as means ± standard deviations. Statistically significant changes are indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to sham or chow control group. Transcriptional expressions of *Star, Hsd3b1, Cyp11b1,* and *Cyp21a1* were elevated in both CBDL and CDCA-fed mice. Changes of mRNA levels in CDCA-fed mice were independent of TGR5. The figure is reproduced from "*Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in*

adrenocortical H295R cells via S1PR2, ERK and SF-1" (125) with permission of Wiley.

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4.2 Bile acids induce adrenal steroidogenesis by induction of its key enzymes *in vivo*

To determine transcriptional effects of bile acids on steroidogenesis-related genes we next performed RT-PCR in the *in vivo* models. CBDL significantly induced adrenal steroidogenesis-related genes such as *Star, Hsd3b1, Cyp11b1, Cyp21a1,* and *Mc2r* expression (Figure 7 C). CDCA feeding also increased mRNA expressions of these genes significantly compared to chow controls. Notably, in CDCA-fed mice, improved transcript abundance of steroidogenesis-related genes were independent of FXR and TGR5 (Figure 7 D and Figure 8 B), which was consistent with our previous findings. A further Western Blotting analysis in adrenal glands of long-term CBDL mice verified the increased protein levels of CYP11B1 and CYP21A1(Figure 7 E).

Taken together, these results strongly argue that bile acids facilitate glucocorticoid secretion and synthesis in CBDL and CDCA-fed mice, which is independent of FXR and TGR5.

4.3 Cholestasis and CDCA feeding lead to loss of cholesterol storage in mouse adrenal glands

It is reported that treatment of FXR agonist GW4064 shall increase plasma glucocorticoid levels in C57BL/6 mice by altering lipid metabolism (126). We therefore detected the mRNA levels of genes related to lipid transport and synthesis. As shown in Figure 9 A and B, the gene encoding ABCA1 that is responsible for efflux of cholesterol was transcriptionally inhibited by CBDL or CDCA-supplemented diet. However, the mRNA level of the gene encoding SR-B1 that transport cholesterol from peripheral blood into cells was elevated under the same situation.

Meanwhile, genes related to cholesterol synthesis such as *Cyp51* and *Hmgcred* were enhanced at transcription level. These results necessitate the measurement of cholesterol concentration in adrenals.

We then conducted measurements of cholesterol concentrations in adrenals of 3-week CBDL mice and CDCA-fed mice. Expectedly, the free cholesterol kept unchanged while the total cholesterol decreased by a large margin in adrenals of CBDL mice compared with sham control group, which was associated with substantially reduced cholesteryl ester. Thus, the decreased cholesteryl ester accounts for the loss of total cholesterol (Figure 9 C). In CDCA-feeding mice, the decreased cholesteryl ester only existed in CDCA-fed wild type mice but not in *Fxr*$^{-/-}$ mice (Figure 9 D)

Since hormone-sensitive lipase (HSL) plays an important role in converting cholesteryl ester into free cholesterol, following Western Blotting assays were conducted. As shown in Figure 9 E, in adrenals of CBDL mice, both the total HSL and phosphorylation of HSL at site 563 advanced dramatically in contrast to sham group. Interestingly, an oil red staining assay in the adrenals of sham and CBDL mice clearly showed that the neutral lipids stained by the oil red shrank substantially (Figure 9 F), which accorded with the decreased cholesteryl ester level in adrenals of CBDL mice.

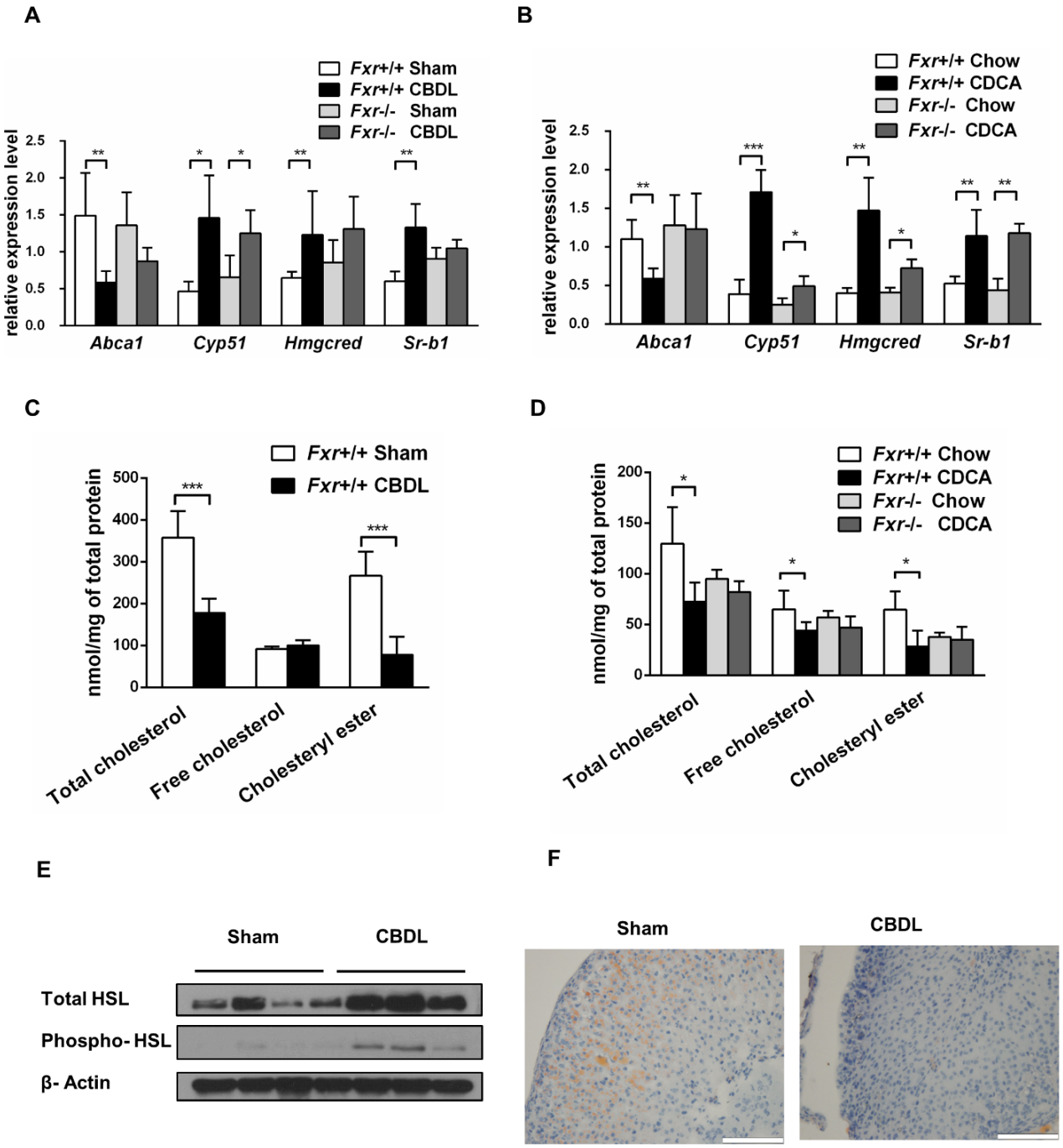

Figure 9 Effect of cholestasis and CDCA feeding on cholesterol storage in mouse adrenals

A, **B**: RNA samples were extracted from adrenal glands of 7-day CBDL and 5-day CDCA-fed mice for RT PCR analysis. All relative mRNA levels of genes were normalized to cyclophilin. mRNA levels of *Abca 1* were decreased while mRNA levels of *Cyp51*, *Hmgcred*, and *Sr-b1* were increased upon CBDL surgery and CDCA feeding. **C**: Adrenals from 3-week sham operated or CBDL mice were subjected to cholesterol measurements with colorimetric

method. Total cholesterol, free cholesterol and cholesteryl ester levels were determined. **D**: *Fxr*$^{+/+}$ and *Fxr*$^{-/-}$ mice were fed with 1% CDCA for 5 days. The adrenals were harvested and cholesterol measurements with colorimetric method were conducted furthermore to determine the concentrations of total cholesterol, free cholesterol and cholesteryl ester. **E**: Cell lysates from adrenals of 3-week CBDL mice were subjected to Western Blotting assays with specific antibodies against total HSL and phosphorylated HSL at 660 site. **F**: Adrenals from 1-week CBDL mice were stored, cryosectioned and stained with Oil Red. Orange spots demonstrated the changes of neural lipid concentrations. Scale bars: 100 μm. For **A-D**, there are 3-4 independent samples per group. Values are presented as means ± standard deviations. Statistically significant changes were indicated by asterisks. * p<0.05, *** p<0.001

4.4 Conjugated bile acids facilitate glucocorticoid synthesis and increase expressions of steroidogenic enzymes in human adrenocortical H295R cells

Since the effects CBDL and CDCA on corticosterone production and gene expression levels *in vivo* may also be caused by factors other than bile acids, at least in part, such as systemic changes in the whole HPA axis, we studied the sole effects of bile acids in a human cell line to rule out such variables at best. We therefore treated human adrenocortical H295R cells with different kinds of bile acids and examined adrenal cortisol secretion *in vitro*. Amongst the bile acids we tested, only conjugated CDCA, especially TCDCA, significantly raised cortisol synthesis (Figure 10 A and B). The other tested bile acids including DCA, TDCA, TCA, and CDCA, however, did not significantly activate cortisol secretion. TCDCA affected cortisol synthesis in a dose dependent manner ranging from 100 to 400 μM in this system (Figure 10 C). MTT assays were performed to evaluate the potential cell toxicity caused by high dose of TCDCA, proving that the tested concentrations of

TCDCA did not cause severe cell toxicity (Fig 10 D). Given that in cholestatic patients total serum bile acid level amounts to 300 μM and CDCA is the most abundant bile acid in humans (121, 122), we chose a dose of 400 μM which produced the most significant effects for the following experiments. Consistent with our results from animal models, 400 μM TCDCA dramatically enhanced mRNA levels of *STAR*, *CYP21A2* (homologue gene of *Cyp21a1* in mice) and *HSD3B2* (homologue gene of *Hsd3b1* in mice) (Figure 10 E). Western Blotting assays furthermore confirmed the elevated protein levels of CYP21A2 and STAR in H295R cells upon TCDCA treatments (Figure 10 F and G), which is consistent with the changes of mRNA levels. As cortisol will be secreted by H295R cells immediately into the media once it is synthesized (127), our data demonstrate that conjugated CDCA can directly enhance glucocorticoid secretion in H295R cells.

We continued to test the involvements of FXR and TGR5 in the effect of TCDCA on H295R cells. Interestingly, we detected the existence of mRNA levels of FXR and TGR5 by RT-PCR (Figure 11 A). FXR protein was also detected in H295R cells by Western Blotting (Figure 11 B). Despite co-existence of FXR and TGR5 in H295R cells, neither the FXR agonist INT-747 nor the TGR5 agonist INT-777 successfully activated cortisol secretion in H295R cells (Figure 12 A), demonstrating again that the observed effects of conjugated bile acids on adrenal steroidogenesis are independent of FXR and TGR5, the two well characterized bile acid receptors.

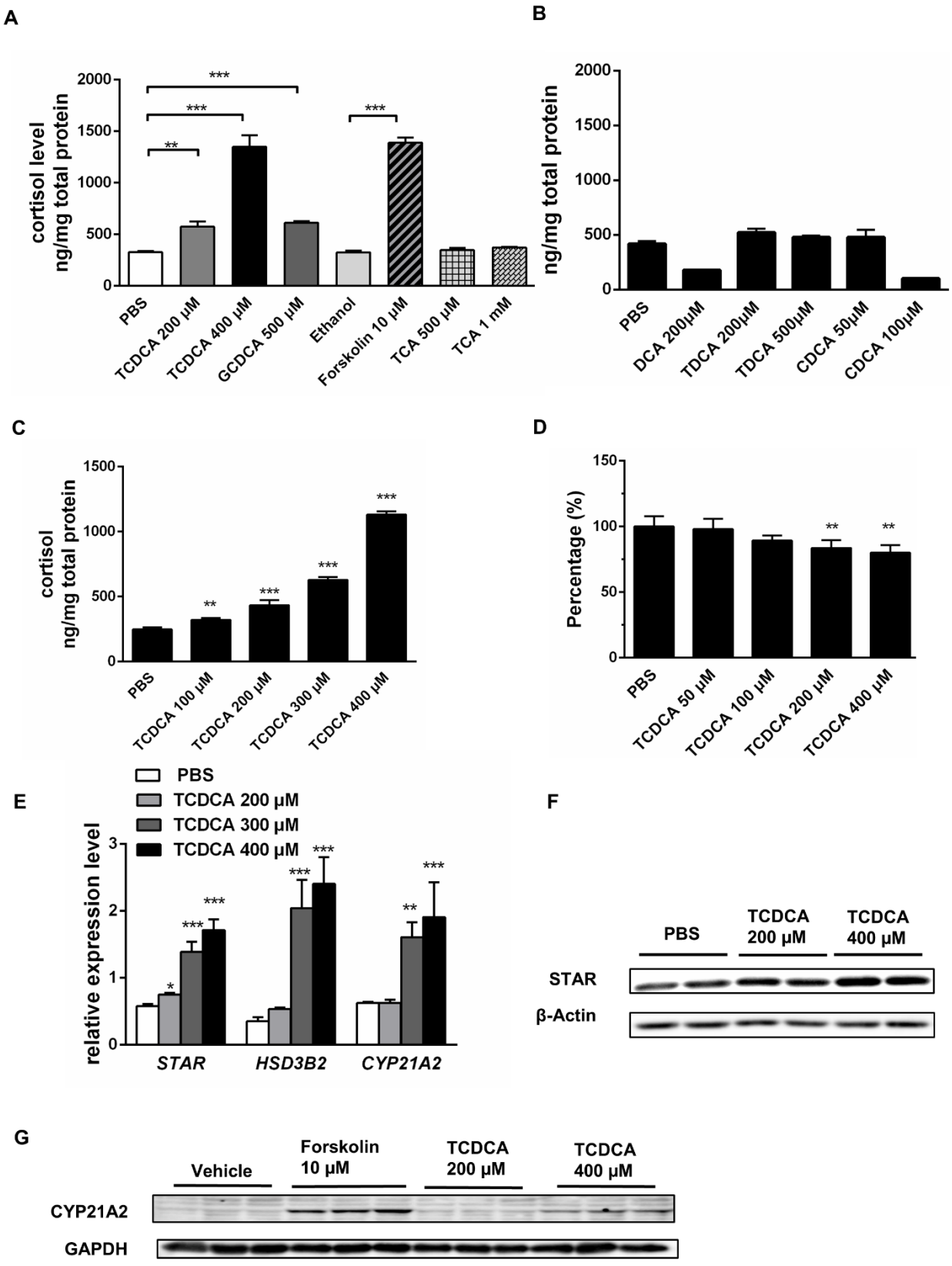

Figure 10 Conjugated bile acids directly induced cortisol secretion and steroidogenesis in H295R cells

A, **B**: Human carcinoma adrenal cell line, H295R cells, were cultured and incubated for 48h

with different kinds of bile acids including DCA, TDCA, CDCA, TCDCA and TCA. Cortisol secreted into the supernatant was measured and normalized to total proteins of cultured cells of each well. Only conjugated CDCA increased cortisol secretion of H295R cells. **C**: H295R were treated for 48h with increasing concentrations of TCDCA from 100 to 400 µM. Cortisol secreted into the supernatant (normalized to total protein of cultured cells) increased in a dose-dependent manner in response to TCDCA challenge**. D**: MTT assays to determine the cell viability rates of different doses of TCDCA-treated H295R cells were performed as described in “Materials and Methods”. Optical density of samples were measured at the wavelength of 570 nm and calculated as the percentages in contrast to the PBS control group. **E**: RNA was extracted from the cells treated with 200-400 µM TCDCA and used for RT PCR assays. Relative mRNA expression levels of related genes were calculated and normalized to housekeeping gene 18S rRNA. *STAR*, *HSD3B2*, and *CYP21A2* were shown to be increased in a dose-dependent manner by TCDCA. For **A**-**E**, there are 3-4 independent samples per group. Values are presented as means ± standard deviations. Statistically significant changes were indicated by asterisks. ** $p<0.01$, *** $p<0.001$. **F**: 200 µM and 400 µM TCDCA treatment for 24 hours in H295R cells led to elevated STAR protein expressions. β-Actin acted as the internal control. **G**: 200 µM and 400 µM TCDCA treatment for 48 hours in H295R cells led to elevated CYP21A2 protein expressions. GAPDH acted as the internal control. 10 µM forskolin, a PKA activator, was added as a positive control. Part **C, D,** and **E** are reproduced from "*Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1"* (125) with permission of Wiley.

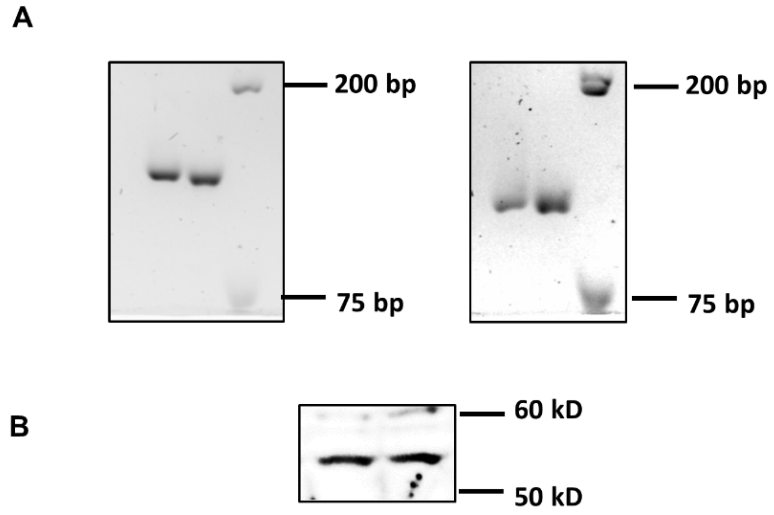

Figure 11 Expressions of FXR and TGR5 in H295R cells

A: RNA samples were extracted from cultured H295R cells. Total RNA samples were subjected to RT PCR with specific primers for *FXR* and *TGR5.* RT PCR products of *TGR5* (left panel) and *FXR (right panel)* were separated by agarose electrophoresis. DNA fragments (128 bp for *TGR5* and 104bp for *FXR* respectively) were detected and visualized by Image Lab software. **B:** Two H295R cell lysate samples were subjected to Western Blotting assays with a primary antibody against total FXR. Clear protein bands were detected between 50 kD and 60 kD (predicted size of the detected FXR protein: 55 kD). The figure is reproduced from "*Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1"* (125) with permission of Wiley.

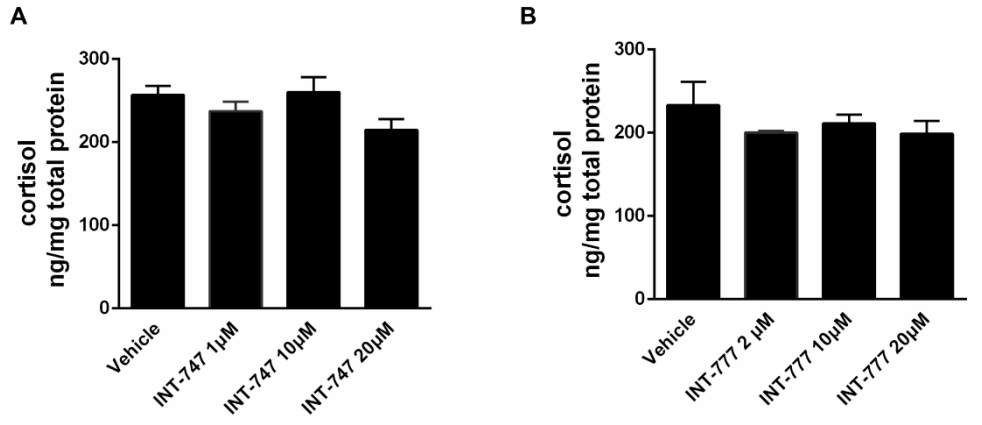

Figure 12 FXR and TGR5 agonists could not activate cortisol secretion in

H295R cells

H295R cells were incubated with FXR and TGR5 agonists (INT-747 and INT-777 respectively) for 48 hours. Cortisol secretion in the supernatant sample was determined, normalized to total protein, and presented as means ± standard deviations. There are 3-4 independent samples per group.

4.5 Bile acid-induced steroidogenesis is not mediated by PKA

Under physiological conditions, MC2R is activated by ACTH binding and stimulates adrenal steroidogenesis via PKA pathway as a result (128). *MC2R* transcription in both animal models and in TCDCA treated cells were elevated and phosphorylation of HSL in CBDL mice increased as well. These results led us to address the potential involvement of PKA pathway in the adrenal regulation by bile acids. To test whether bile acid-induced cortisol secretion in H295R is PKA-dependent, we preincubated the H295R cells with various concentrations of Rp-isomer acting as a PKA inhibitor. While the mild increased intracellular cAMP and phosphorylation of HSL as a PKA substrate were detected in TCDCA-treated H295R cells (Figure 13 A and B), neither H295R cells treated by Rp-isomer showed substantial reduction of cortisol secretion caused by TCDCA (Figure 13 C), nor did Rp-isomer influence the agonistic effect of TCDCA on mRNA expressions of *STAR*, *CYP21A2* and *HSD3B2* (Figure 13 D). Absent effects of Rp-isomer on cortisol production also exclude a prominent role for TGR5 in our models, since TGR5 acts via PKA (61). Combined with the data that incubation of H295R cells with TGR5 agonist INT-777 did not stimulate cortisol secretion (Figure 12), these data widely exclude TGR5-PKA-dependent bile acid-induced cortisol secretion in the used model systems.

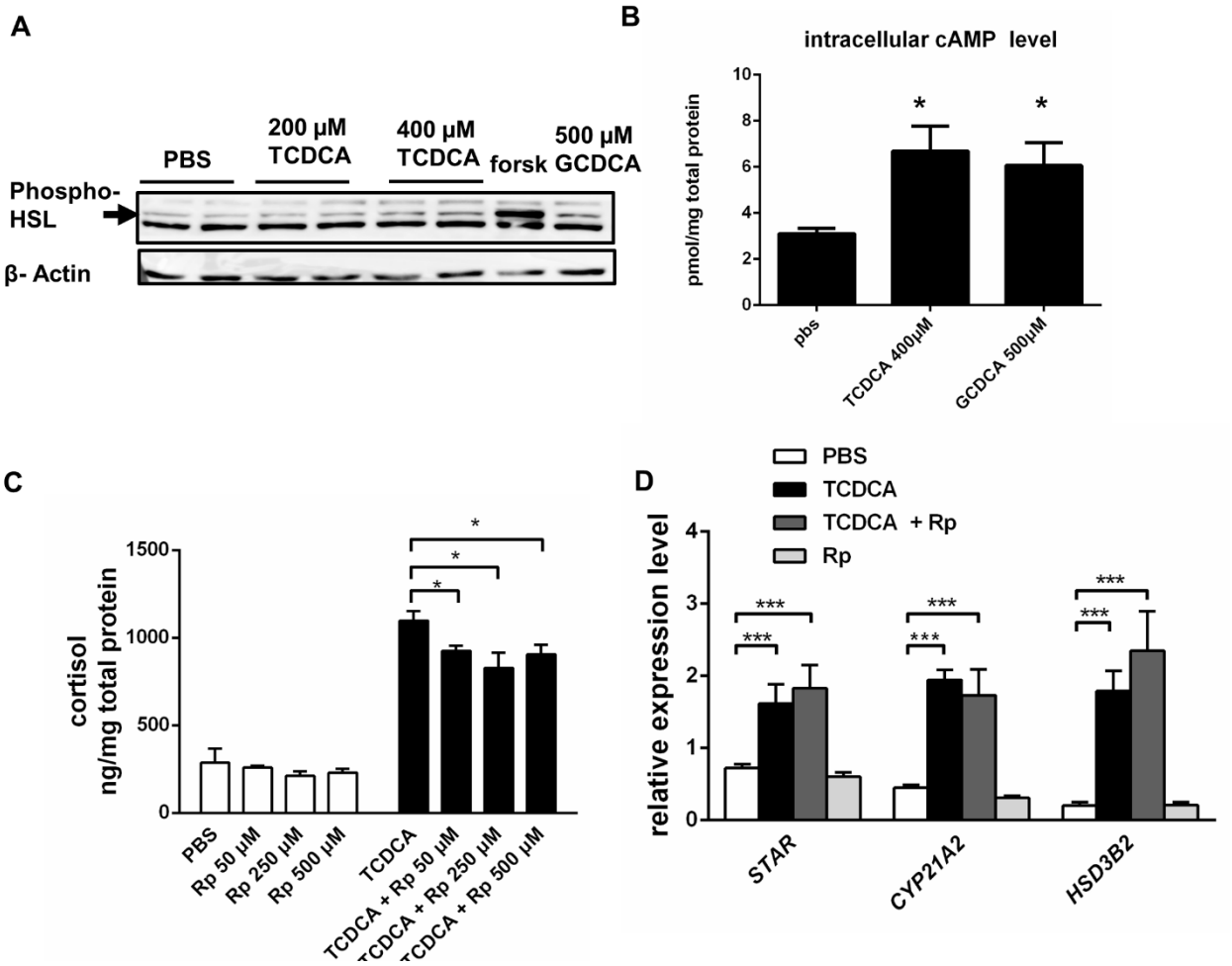

Figure 13 TCDCA-induced cortisol secretion is independent of PKA activity in H295R cells

A, B: H295R cells were incubated with different concentrations of conjugated CDCA for 15 minutes (**A**) or 40 minutes (**B**). Then cells were washed, harvested with lysis buffer and subjected to Western Blotting with a specific antibody against phosphorylated HSL at 660 site (**A**) or intracellular cAMP measurements (**B**). 10 µM forskolin acted as a positive control of PKA activator. N=3-4 per group. Values are presented as means±SD and asterisks indicate statistically significant changes. * $p<0.05$. **C, D**: H295R cells were treated with 400 µM TCDCA. 50-250 µM Rp-Isomer (a PKA inhibitor) was added and cells were treated for 48 hours. There are 3 independent samples in each group. Rp-Isomer reduced TCDCA-induced cortisol secretion only to a minor extent (**C**). Co-Treatment with Rp-Isomer (250 µM) had no effect on TCDCA-mediated induction of *STAR*, *CYP21A2* and *HSD3B2* mRNA levels (**D**). mRNA levels were normalized to 18S rRNA. Values are presented as means ± standard

deviations. Asterisks indicate statistically significant changes. **C**: * $p<0.05$, compared to TCDCA treatment group. **D**: *** $p<0.001$, compared to PBS control. Part **C** and **D** are reproduced from "*Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1"* (125) with permission of Wiley.

4.6 TCDCA activates ERK phosphorylation in mouse adrenals and H295R cells and ERK phosphorylation is crucial to bile acid-induced cortisol secretion

MAPK/ERK signaling pathway has been uncovered to be closely related to steroidogenesis in addition to cAMP-PKA pathway (129). Therefore, we next studied the role that phosphorylation of ERK plays in the regulation of conjugated bile acid on steroidogenesis. To begin with, the mouse adrenals were *ex vivo* cultured and incubated with 200 μM TCDCA for 2 hours (Figure 14 A). Intriguingly, phosphorylation of ERK was detected and enhanced substantially upon TCDCA treatment. H295R cells were then cultured *in vitro* and incubated with varying doses of TCDCA. As shown in Figure 14 B, treatments of 50 μM to 200 μM TCDCA also enhanced ERK phosphorylation as assessed by Western Blotting analysis. In contrast, when 10 μM MEK1/2 inhibitor U0126 was added together with 400 μM TCDCA, the strikingly elevated phosphorylation of ERK caused by TCDCA was totally blocked, proving that phosphorylation of ERK was activated by MEK/MAPK pathway (Figure 14 C and D).

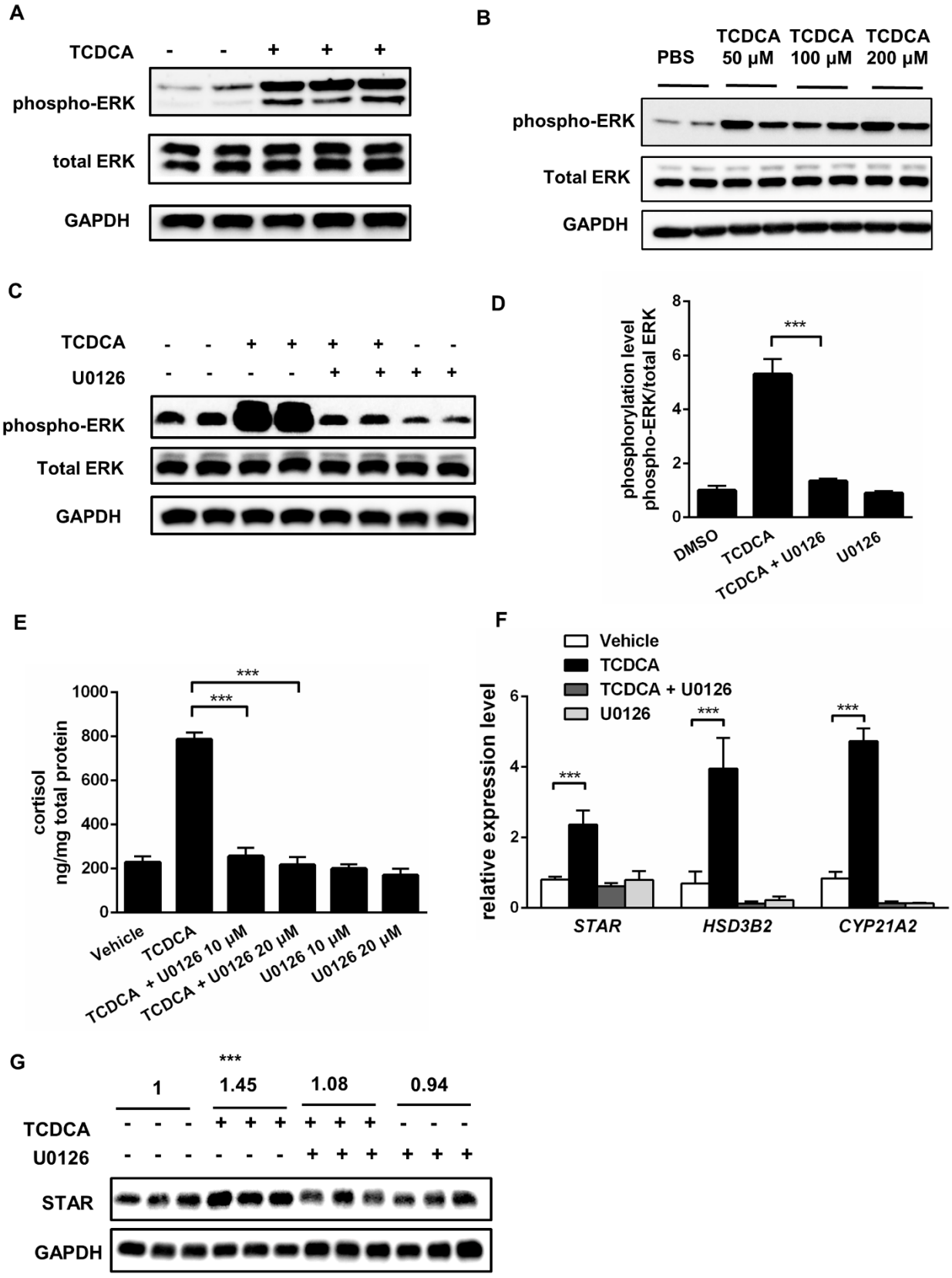

Figure 14 TCDCA-induced cortisol secretion and synthesis depended on ERK phosphorylation.

A: Mouse adrenals were cultured *ex vivo* and treated with 200 µM TCDCA for 2 hours. Western Blotting assays demonstrated significantly induced phosphorylation of ERK in TCDCA-treated adrenals compared to untreated controls. **B**: H295R cells treated with 50, 100, and 200 µM TCDCA for 48h were harvested and subjected to Western Blotting assays. Phosphorylation of ERK was elevated in TCDCA treated cells. **C-F**: U0126, a specific MEK inhibitor blocking phosphorylation of ERK were added into cell culture assays. H295R cells were challenged with 400 µM TCDCA and 10 or 20 µM U0126 for 24h (**C**, **D**) or 48h (**E**-**F**). TCDCA substantially induced phosphorylation of ERK and U0126 specifically inhibited ERK phosphorylation caused by TCDCA (**C**, **D**). Cortisol secretion was no longer activated by 400 µM TCDCA once 10 or 20 µM U0126 was added (**E**). Meanwhile, 10 µM U0126 successfully abolished increased transcription abundance of *STAR*, *HSD3B2*, and *CYP21A2* by 400 µM TCDCA (**F**). mRNA levels were normalized to 18S rRNA. Values are presented as means ± standard deviations. Statistically significant changes are indicated by *** $p<0.001$, compared to DMSO (**F**) or TCDCA treatment (**D**, **E**). There 3-4 independent samples per group. **G**: H295R cells were incubated with 400 µM TCDCA and 10 µM U0126 for 48 hours. Cell lysates were subjected to Western Blotting assays. Averages of quantified band densities related to the vehicle control group were shown. Statistically significant changes are indicated by asterisks compared to the vehicle control group, *** $p<0.001$. The figure is adapted from "*Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1"* (125) with permission of Wiley.

Meanwhile, when H295R cells were co-incubated with 10 µM U0126 and 400 µM TCDCA, cortisol secretion was strongly inhibited compared to the treatment of 400 µM TCDCA alone (Figure 14 E). Consistent with cortisol secretion data 10 µM U0126 also successfully abolished the upregulation of mRNA levels of *STAR*, *HSD3B2*, and *CYP21A2,* demonstrating that TCDCA failed to enhance the transcription abundance of steroidogenesis-related genes given the existence of

U0126 (Figure 14 F). Further Western Blotting assays also showed that STAR protein levels increased to about 1.5 times by 400 μM TCDCA compared to vehicle group, while its protein levels could not be upregulated by 400 μM TCDCA when 10 μM U0126 was added (Figure 14 G).

Taken together, these findings demonstrate that conjugated CDCA successfully activates ERK phosphorylation in mouse adrenals *ex vivo* and human H295R cells *in vitro.* Phosphorylation of ERK is indispensable for conjugated bile acid-induced steroidogenesis in adrenal glands and thus plays a crucial role in regulation of conjugated bile acids, since blocking ERK phosphorylation inhibited bile acid-induced cortisol secretion in human H295R cells.

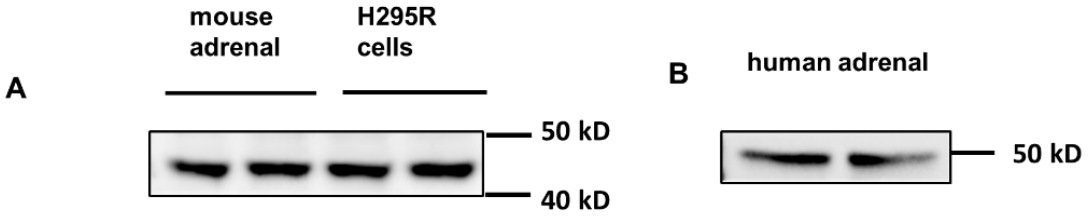

Figure 15 Expression of S1PR2 in adrenocortical cells

Western Blotting assays were conducted in mouse adrenals, H295R cells, and snap-frozen human adrenals from organ-donating volunteers with S1PR2-specific antibody. Clear protein bands were observed between 40 kD and 50 kD in murine adrenal cortical samples, and nearer to 50 kD in human adrenal samples. The figure is reproduced from "*Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1"* (125) with permission of Wiley.

4.7 S1PR2 modulates enhanced steroidogenesis caused by conjugated bile acids

S1PR2 is a novel bile acid receptor characterized in mouse liver and proven to lead to phosphorylation of ERK once it is bound and activated by conjugated bile acids

(65). We first confirmed the expression of S1PR2 in adrenocortical tissues. Clear protein bands were detected between 40 kD and 50 kD in Western Blotting assays (Figure 15). To study if the effect of conjugated bile acids on adrenal steroidogenesis is mediated by S1PR2 in H295R cells, we used a specific S1PR2 antagonist, JTE-013, in a loss-of-function experiment. H295R cells were incubated with 10 μM JTE-013 and 400 μM TCDCA together, for 48 hours. We found that increased phosphorylation of ERK induced by TCDCA compared to vehicle control dropped dramatically from about 5 folds to about 1.4 folds (Figure 16 A and B). As S1PR2 is coupled to multiple types of G proteins including $G\alpha_{q/11}$, a specific inhibitor of $G\alpha_{q/11,}$ YM254890 was applied. Co-incubation of 5 μM YM254890 and 200 μM TCDCA also successfully attenuated phosphorylation of ERK (Figure 16 C). Based on previous results, it is natural to deduce that S1PR2 regulates conjugated bile acids effect via ERK. The following experiments verified the deduction by showing that increasement of cortisol secretion in H295R cells almost diminished when cells were co-incubated with TCDCA and JTE-013 (Figure 16 D). mRNA levels of *STAR*, *HSD3B2*, and *CYP21A2,* in H295R cells were almost unchanged by 400 μM TCDCA with co-incubation of 10 μM JTE-013 in contrast to the treatment of 10 μM JTE-013 alone (Figure 16 E). Western Blotting assays also confirmed that increased levels of STAR protein caused by TCDCA was abolished by JTE-013 (Figure 16 F), which also accorded with the RT PCR results.

More evidence of the involvement of S1PR2 in the regulation of conjugated bile acid in H295R cells were unraveled by additional genetic loss of function experiments. Specific S1PR2 siRNA sequences were transfected into H295R cells. As shown in Figure 17 A, transfection of S1PR2 siRNA resulted in about 50% knockdown of *S1PR2* mRNA level. S1PR2 protein expression level were also decreased by 55% on average (Figure 17 B). As a result of siRNA interference, in specific S1PR2 siRNA-transfected H295R cells, phosphorylation of ERK induced by TCDCA was significantly lowered by 60% (from 18.8-fold to 6.5-fold) as compared to the control

siRNA-transfected cells with TCDCA (Figure 18 A and B). In addition, TCDCA-induced cortisol secretion in siRNA-transfected cells was also reduced significantly (Figure 18 C). Enhanced mRNA levels of *STAR*, *HSD3B2*, and *CYP21A2* due to treatment of TCDCA were alleviated when interference of S1PR2 was introduced (Figure 18 E), which is in line with our chemical inhibitor experiments and further supports the role of S1PR2.

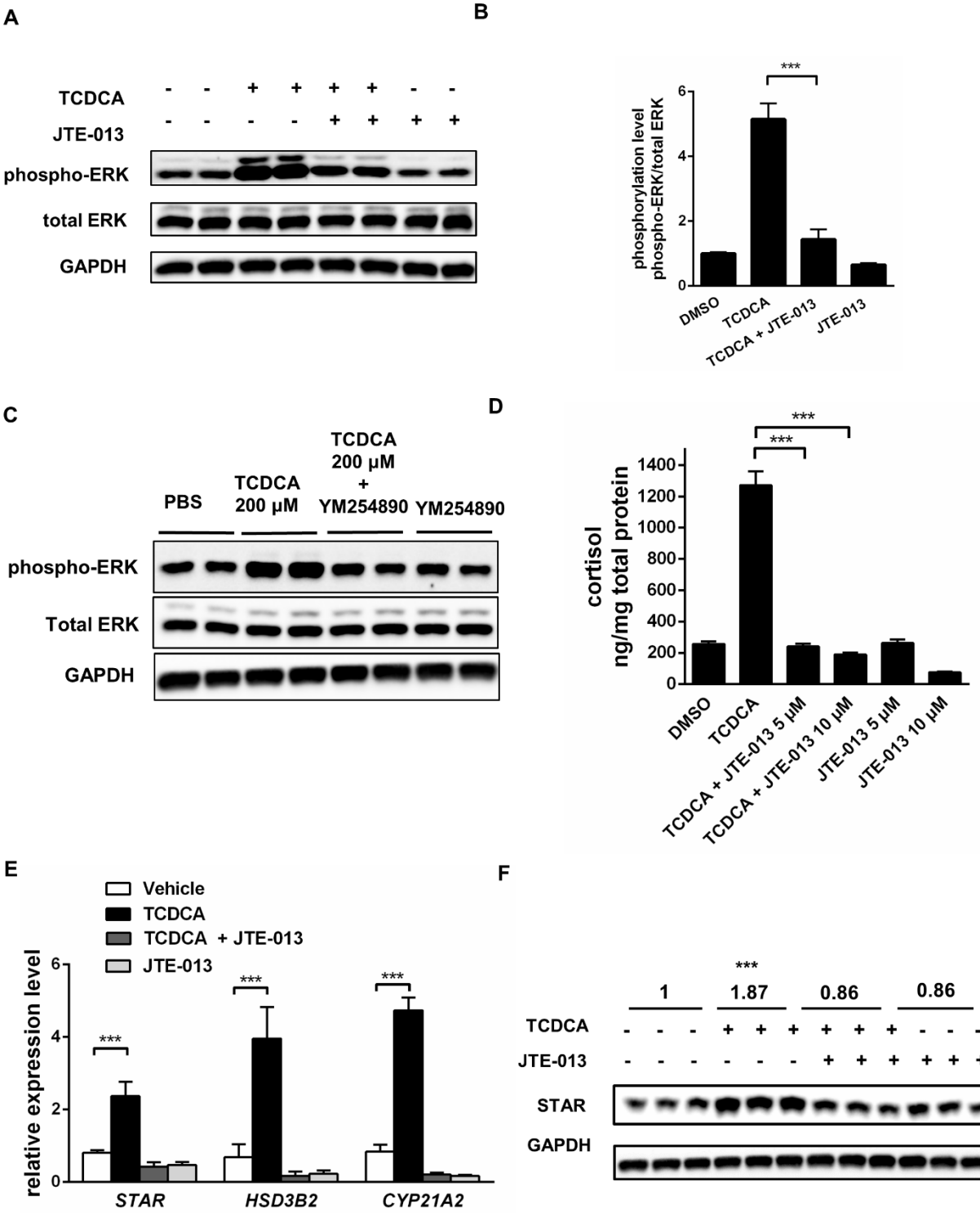

Figure 16 Blocking S1PR2 signaling abolished the ERK phosphorylation, cortisol secretion and steroidogenesis caused by TCDCA in H295R cells

H295R cells were co-incubated with 200 or 400 μM TCDCA for 2 days. 5 and 10 μM JTE-013 (a specific S1PR2 antagonist) and YM254890 (a specific inhibitor of $G\alpha_{q/11}$) were added where indicated. **A**, **B**: H295R cells were incubated with 400 μM TCDCA and 10 μM JTE-013 for 48 hours. Cell lysates were subjected to Western Blotting assays.

Phosphorylation of ERK were detected, quantified, and normalized to corresponding total ERK level. Results indicated that 10 μM JTE-013 strongly suppressed TCDCA-induced ERK phosphorylation. **C**: 200 μM TCDCA were incubated with 5 μM YM254890 for 48 hours. Cell lysates were subjected to Western Blotting assays. Enhanced phosphorylation of ERK induced by 200μM TCDCA were shown to been attenuated by 5 μM YM254890. **D**: H295R cells were incubated with 400 μM TCDCA and 5-10 μM JTE-013 for 48 hours as indicated. Cortisol secreted into the medium were measured and normalized to total protein. Results demonstrated both 5 and 10 μM JTE-013 significantly suppressed enhanced cortisol secretion by 400 μM TCDCA. **E**: H295R cells were incubated with 400 μM TCDCA and 10 μM JTE-013 for 48 hours. RNA samples were extracted for RT PCR analysis. Relative expressions of genes were normalized to 18S rRNA. STAR protein was detected and quantified. mRNA levels of *STAR*, *CYP21A2*, and *HSD3B2* were no longer increased by 400 μM TCDCA given the existence of 10 μM JTE-013. **F**: H295R cells were incubated with 400 μM TCDCA and 10 μM JTE-013 for 48 hours. Protein lysates were harvested for Western Blotting assays. STAR protein increased by 400 μM TCDCA was totally inhibited by JTE-013. Averages of quantified band densities related to the vehicle control group were shown. Statistically significant changes are indicated by asterisks, compared to the vehicle control group. For **B**, **D**, **E**, there are 3-4 independent samples per group. Values are presented as means ± standard deviations. Statistically significant changes are indicated by asterisks, compared to TCDCA treatment group (**B**, **D**) or vehicle control (**E**). *** $p<0.001$. Part **A**, **B**, **D**, **E**, and **F** are reproduced from "*Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1"* (125) with permission of Wiley.

Taken together, these findings suggest that S1PR2-ERK pathway mainly mediates conjugated BAs-induced steroidogenesis steroidogenic cells.

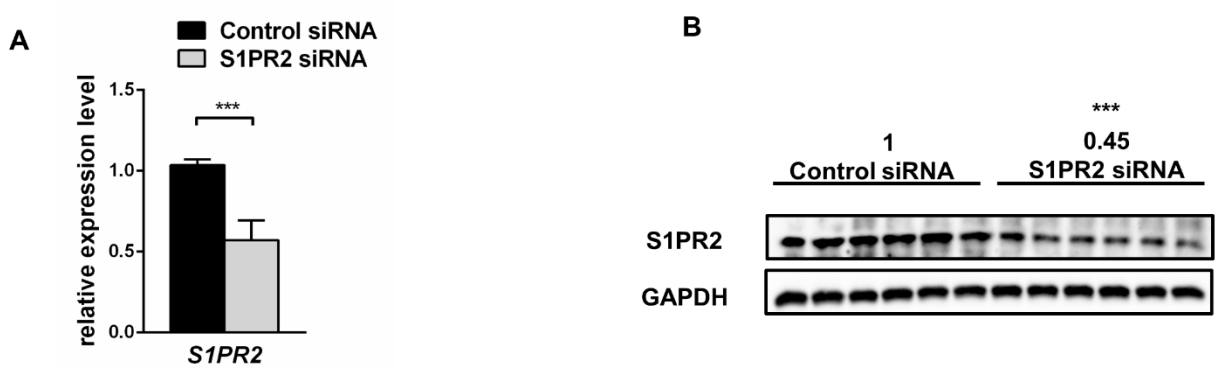

Figure 17 Expression levels of S1PR2 after siRNA interference

Specific S1PR2 siRNA oligos were transfected into H295R cells as described in "Materials and Methods". After 48 hours incubation with siRNA, H295R cells were harvested for RT PCR analysis (**A**) and Western Blotting assays (**B**) to determine changes of S1PR2 expression. **A**: mRNA levels of S1PR2 were normalized to 18S rRNA. Values are presented as means ± standard deviations. Statistically significant changes are indicated by asterisks, compared to control siRNA group. *** $p<0.001$. **B**: S1PR2 protein was detected and quantified. GAPDH acted as the internal control. Averages of quantified band densities related to the control group were shown. Statistically significant changes are indicated by asterisks, compared to the vehicle control group. *** $p<0.001$. The figure is adapted from "*Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1"* (125) with permission of Wiley.

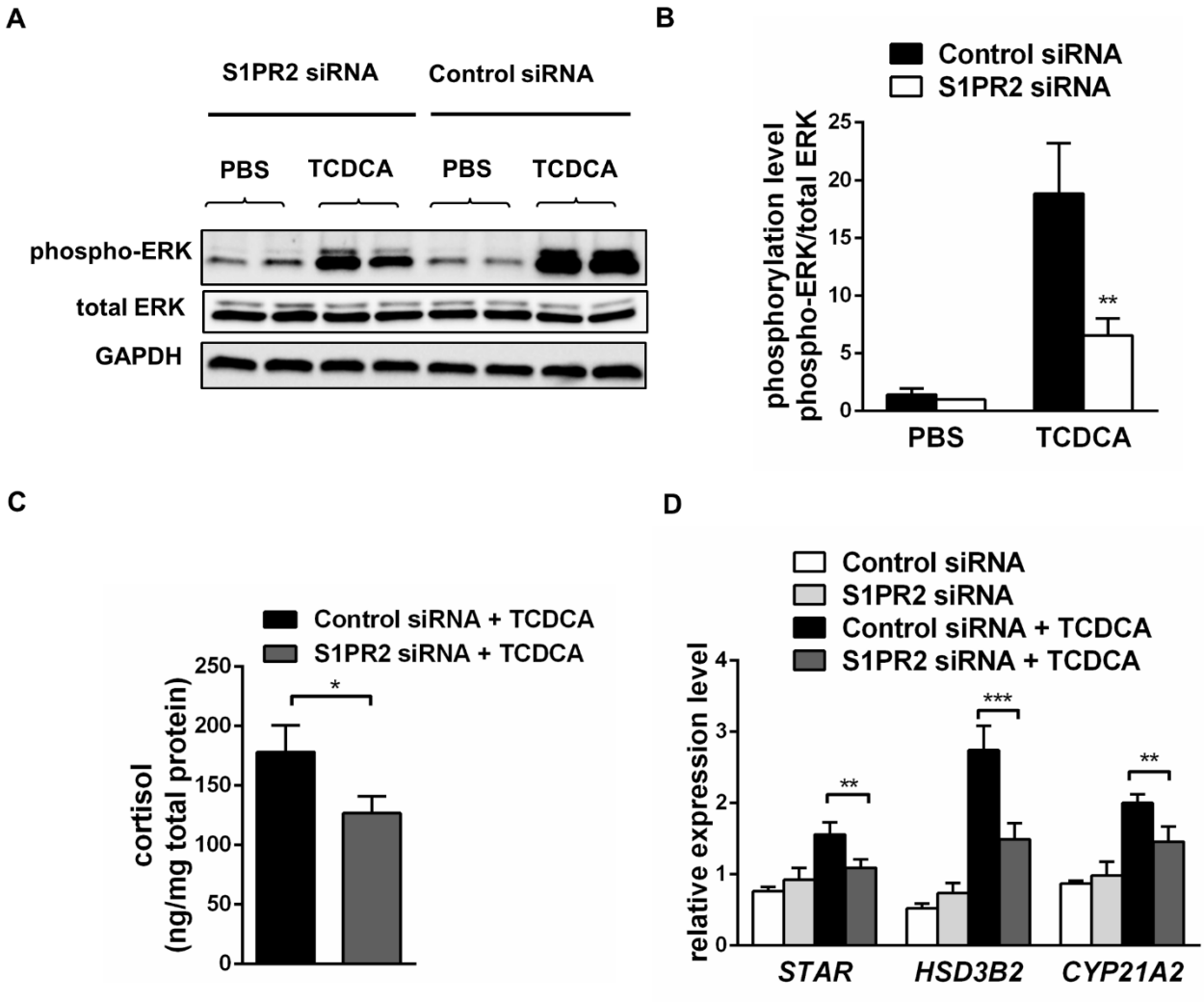

Figure 18 Interference of S1PR2 siRNA attenuated enhanced phosphorylation of ERK and steroidogenesis by TCDCA in H295R cells

Oligos of siRNA specific to S1PR2 together with the negative control were transfected into H295R cells for 48 hours. Then the transfection medium was removed and H295R cells were incubated with complete growth medium containing 400 μM TCDCA for 48 hours. **A**, **B**: Cell lysates were harvested and subjected to Western Blotting assays. Phosphorylation of ERK was detected, quantified, and normalized to corresponding total ERK level. Increasement of phosphorylation of ERK caused by TCDCA was substantially attenuated when S1PR2 siRNA was transfected. **C**: Medium samples were collected for cortisol measurements. Cortisol secreted into the medium was normalized to the total protein of each well. Increasement of cortisol secretion induced by TCDCA was reduced upon S1PR2 siRNA transfection. **D**: RNA samples were extracted for RT PCR assays. Relative

expression levels of genes were normalized to 18S rRNA. Increasements of transcription abundance of *STAR*, *HSD3B2*, and *CYP21A2* by TCDCA were significantly reduced after S1PR2 siRNA was transfected. For **B**, **C**, **D**, there are 3-4 independent samples per group. Values are presented as means ± standard deviations. Statistically significant changes are indicated by asterisks, compared to control siRNA group alone (B), or control siRNA plus TCDCA treatment group (**C**, **D**). *p<0.05, ** p<0.01, *** p<0.001. The figure is adapted from "*Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1"* (125) with permission of Wiley.

4.8 Conjugated bile acid facilitates steroidogenesis in H295R cells by promoting SF-1 transactivation activity

The transcription factor SF-1 is a transcription factor regulating steroidogenesis in adrenocortical tissues. It is phosphorylated by ERK and once phosphorylation, its transactivation activity is significantly enhanced (130). To study if SF-1 takes parts in induction of steroidogenesis by conjugated bile acids, we next designed luciferase activity assays. As SF-1 is endogenously expressed in H195R cells, we did not overexpress SF-1 artificially in H295R cells. A luciferase reporter plasmid with SF-1 responsive elements was transfected into H295R cells. Once SF-1 is activated and bound to the promoter region of luciferase gene, expression of luciferase will be increased and detected by luciferase reactions. As shown in Figure 19 A, increasing doses of TCDCA from 200 to 400 μM gradually increased luciferase activities, reflecting enhanced SF-1 transactivation activities. AC45594, a SF-1 inverse agonist was applied into cell culture experiments and H295R cells were incubated with AC45594 and TCDCA. As shwon in Figure 19 B, 60 μM AC45594 reduced TCDCA-induced cortisol secretion by 65%. Addition of 60 μM AC45594 also substantially diminished increased mRNA levels of *STAR, HSD3B2,* and *CYP21A2* (Figure 19 C). Intriguingly, as the important regulator, SF-1 itself is affected by conjugated bile acid as well. As shown in Figure 19 D and E, the basal SF-1 protein

levels were slightly increased upon treatment of 400 μM TCDCA. Meanwhile, 10 μM S1PR2 inhibitor JTE-013 reduced SF-1 protein levels by 70% (Figure 19 D). When ERK phosphorylation was blocked by 10 μM U0126, SF-1 protein levels were also dramatically reduced by 60% (Figure 19 E).

Taken together, these data indicate that conjugated bile acids facilitate steroidogenesis in H295R cells by promoting SF-1 transactivation activity and SF-1 acts as a vital mediator linking S1PR2-ERK signaling and steroidogenesis process.

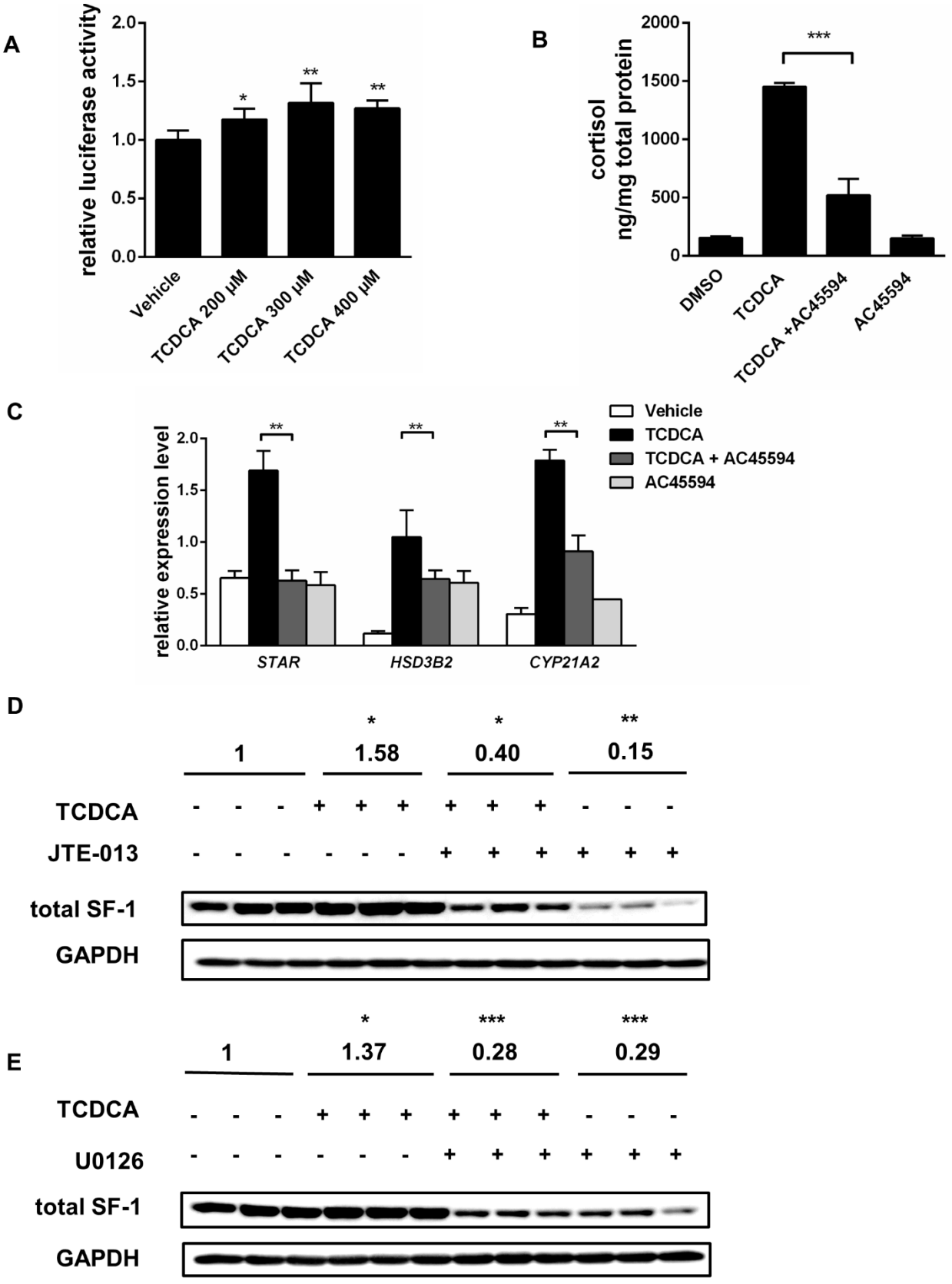

Figure 19 SF-1 transactivation activity and protein level were affected by TCDCA in H295R cells

A: Luciferase reporter plasmid were transfected into H295R cells for 48 hours. After that, cells were cultured with 200-400 μM TCDCA for 48 hours before harvesting. Luciferase activity were measured by a chemiluminescent detection kit. Results were normalized to β-galactosidase activity as the internal control. Transactivation activity of SF-1 was gradually increased by 200-400 μM TCDCA. **B, C**: 60 μM AC45594 as a specific SF-1 inverse agonist was added together with 400 μM TCDCA to incubate H295R cells for 48 hours. Medium samples were collected for cortisol measurements (**B**) and RNA samples were extracted for mRNA analysis (**C**). Cortisol secreted into the medium was normalized to the total protein of each well. Addition of AC45594 successfully inhibited cortisol secretion caused by TCDCA. Relative expression levels of genes were normalized to 18S rRNA. Increasements of transcription abundance of *STAR*, *HSD3B2*, and *CYP21A2* by TCDCA were blocked with the addition of AC45594. **D**, **E**: 10 μM JTE-013 or U0126 were added together with 400 μM TCDCA to incubate H295R cells for 48 hours. Protein lysates were harvested for Western Blotting assays. GAPDH was used as the internal control. Averages of quantified band densities related to the vehicle control group were shown. Asterisks indicate statistically significant changes, compared to the vehicle control group. JTE-013 and U0126 treatment substantially inhibited SF-1 protein expressions. For **A**, **B**, **C**, there are 3-4 independent samples each group. Values are presented as means ± standard deviations. Statistically significant changes are indicated by asterisks, compared to vehicle group (**A**) or TCDCA treatment group (**B**, **C**). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. The figure is adapted from "*Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1"* (125) with permission of Wiley.

5. Discussion

Despite the regulatory role of bile acids in the brain and liver, the question whether bile acids directly affect glucocorticoid synthesis in the adrenal gland has not been well elucidated by other researchers before. We have herein shown that BAs can trigger MEK-ERK-SF-1 signaling via S1PR2 as summarized schematically in a proposed model in Figure 20. Phosphorylated ERK thus phosphorylates and activates SF-1 which regulates the transcription of a wide range of steroidogenesis-related genes including *STAR*, *HSD3B*, and *CYP21*. Our data demonstrate for the first time, that conjugated bile acids can directly influence adrenal glucocorticoid production.

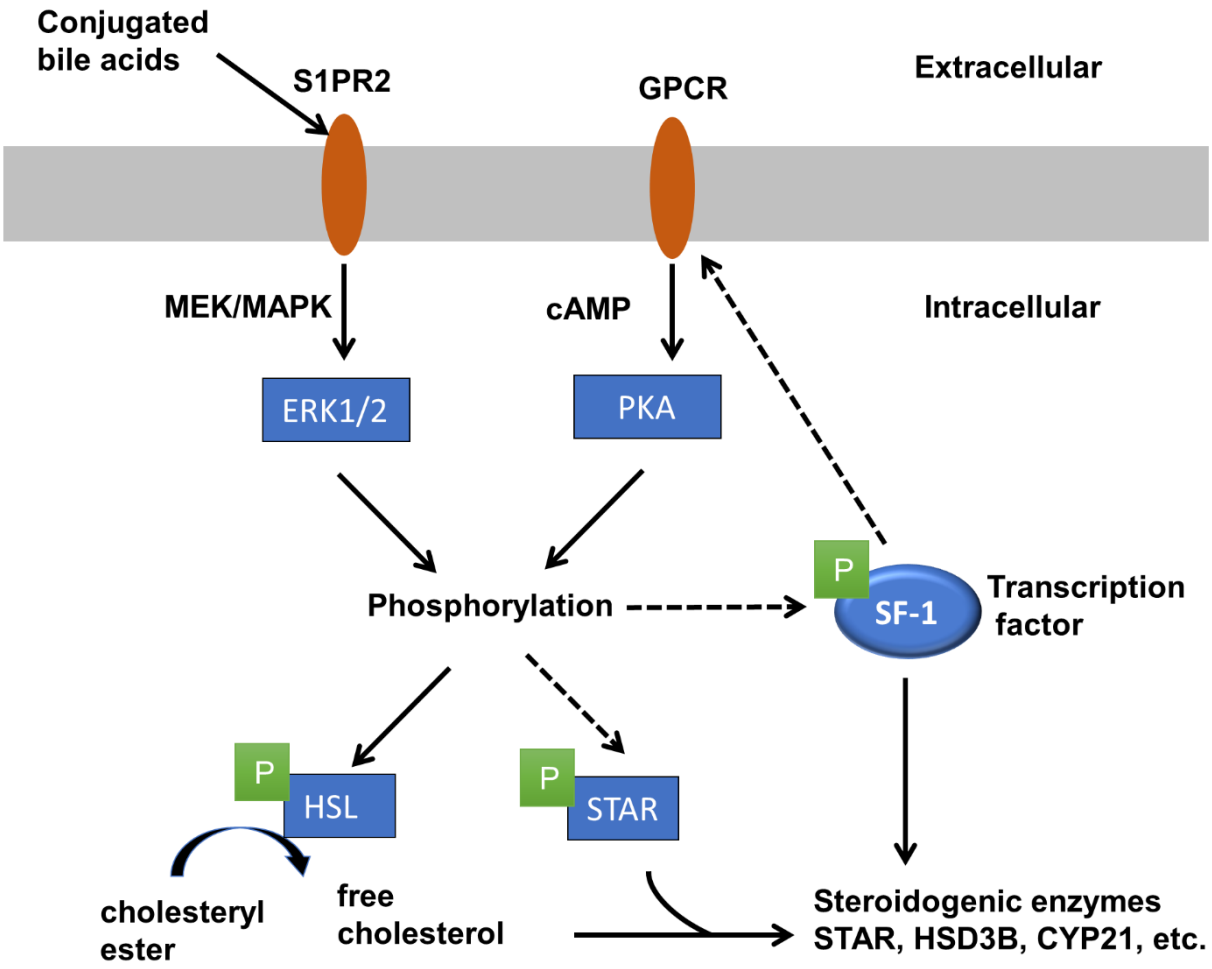

Figure 20 Schematic of proposed signaling pathways

All involved pathways were summarized and presented. Arrows with solid lines stand for

signaling pathways tested and verified in this thesis. Arrows with dotted lines represent those proposed signaling pathways in literature, which, however, has not been tested or confirmed in our system.

5.1 Roles of FXR and TGR5 in regulation of adrenal steroidogenesis

FXR, the most commonly studied nuclear receptor that bile acids bind, has been shown to be expressed in adrenal tissues (117), thus there exists conjectures for long time that bile acids may regulate adrenal function via FXR (131). It is reported that treatment of FXR agonist GW4064 shall increase plasma glucocorticoid levels and reduce adrenal cholesteryl ester level in C57BL/6 mice by altering lipid metabolism (126). Other studies also indicate that FXR blocks the expression of ABCA1 via microRNA-144 (132) and directly facilitates SR-BI expression (133) in hepatocytes. Similarly, in our study, we also observed a decrease of adrenal cholesteryl ester indicating increased need for free cholesterol for cortisol production, which is FXR-dependent in CDCA-fed mouse model (Figure 9). Interestingly, several genes related to *de novo* synthesis of cholesterol were enhanced in adrenals of CBDL and CDCA-fed mice. Meanwhile, SR-B1 mediating the selective uptake of cholesteryl ester was elevated while ABCA1 mediating the selective efflux of cholesterol was inhibited upon excessive bile acid accumulations in our experiments, which are consistent with previous studies. Especially, the influences of CBDL and CDCA-feeding on ABCA1 were dependent of FXR, which may explain the fact that loss of cholesteryl ester is dependent on FXR in CDCA-fed mouse model. These results suggest that FXR may affect steroid metabolism in adrenal glands by altering cholesterol homeostasis. However, both CBDL and CDCA-fed FXR knockout mice developed hypercortisolism. Application of FXR agonist INT-747 also failed to increase cortisol secretion in mice or H295r cell (Fig .12). Meanwhile, most steroidogenesis-related genes, which are affected by bile acids,

are also FXR-independent in mouse model, especially in CDCA-fed mice. In the previous study (131), activation of FXR by GW4064 failed to induce steroidogenesis in mouse adrenals, either. Thus, it is plausible to postulate that, despite its potential role in lipid metabolism, FXR is not the mediator that plays a dominating role in mediating bile acids in the regulation on adrenal glucocorticoid synthesis.

We then tested whether the alternative candidate TGR5, a membrane-bound bile acid receptor expressed in adrenal cortex cells (61, 118), could mediate the observed effects of bile acids in adrenals. First, a CDCA-supplemented diet increased serum corticosterone levels of TGR5 knockout and wild-type mice to similar extents. Meanwhile, the administration of TGR5 agonist INT-777 also failed to booster cortisol secretion in H295R cells *in vitro* (Figure 12 B).

Therefore, the experimental results we have obtained so far demonstrate that neither FXR nor TGR5 is decisive in the effects of bile acids on adrenal steroidogenesis.

Many differences between *Fxr*$^{-/-}$ and *Fxr*$^{+/+}$ mice in response to cholestasis or bile acid challenge (e.g., differences in regard to cytokine response, intestinal permeability, size of bile infarcts, degree of single cell necrosis, liver fibrosis, hepatic corticosterone metabolism) have been reported before and potentially impact on our *in vivo* findings (113, 134-137). It should be noted that in animal models, FXR or TGR5 signaling cascades are not the only factors to determine the effects of bile acids. Therefore, we tried to extend our in *in vivo* findings to *in vitro* assays by culturing H295R cells in order to nail our experiments down to the direct and first-handed effect of bile acids on adrenals. In addition, we used high concentration of conjugated CDCA *in vitro* assays. That is because CDCA is one major bile acid species in humans. More importantly, under cholestatic diseases, the circulating bile acids are not at physiologic concentration, but at supraphysiologically high

concentration, which necessitates the application of pathophysiologically relevant high concentrations of bile acids.

5.2 Influence of HPA axis and PKA activity in adrenals by bile acids

The cAMP-PKA pathway has been reported widely to be engaged in the regulation of steroidogenesis in adrenals. This is mainly via ACTH/MC2R pathway. It has been well established in literature that ACTH treatment leads to elevated transcript level of CYP11A1, CYP17A, CYP11B, and CYP21 (88-92) by activating PKA signaling via the ACTH receptor. STAR is not only affected by CREB at the transcriptional level (138) but also phosphorylated by PKA resulting in elevated enzyme activity (93). Cholesterol synthesis is also affected by PKA signaling (87). ACTH elevates both the transcription and phosphorylation of HSL via PKA pathway (139, 140). We therefore tested whether the observed bile acid effects on adrenal steroid synthesis and secretion are PKA dependent. From the data of our mouse model and human adrenal cell culture model, we observed that phosphorylation of HSL, which is a specific substrate of PKA in mouse adrenals and human H295R cells, was slightly increased. In addition, when H295R cells were treated with conjugated CDCA, the intracellular cAMP levels were increased mildly (Figure 13 B). However, the treatment of a PKA inhibitor Rp-Isomer could not dramatically decrease conjugated CDCA-induced cortisol secretion. TGR5 is associated with $G_{\alpha s}$ protein and increases intracellular cAMP as well once bound by bile acids (61, 118). Application of TGR5 agonist INT-777, however, failed to facilitate cortisol secretion level significantly in H295r cells, either (Figure 12). What's more, transcription of steroidogenesis-related genes was unaffected by PKA inhibition (Figure 13). Thus, we indicate that cAMP/PKA, despite mediating the physiologic response to ACTH, is not a major pathway involved in bile acid-mediated cortisol secretion. The slightly elevated PKA activity is probably due to the expression of some GPCRs increased by phosphorylation of ERK and SF-1.

5.3 Importance of S1PR2 and ERK signaling pathways in the regulation of adrenal steroidogenesis

Among the characterized bile acids receptors, S1PR2 is the novel receptor, which was bound and activated by conjugated bile acids. The signaling of S1PR2 by conjugated bile acids has not yet been fully elucidated and mainly based on livers (65, 71, 141). In our findings, for the first time, we confirm the vital role of S1PR2 in the regulation of conjugated CDCA on adrenal steroidogenesis. The physiological importance of our experiments testing the effects of conjugated bile acids is related to the fact that most bile acids in serum are conjugated *in vivo* (123, 142). Even more importantly, serum concentrations of conjugated bile acids rise dramatically in cholestatic patients compared with normal controls (142), of which the GCDCA and TCDCA together constitute the major part of conjugated BAs. However, we currently still lack direct evidence proving that TCDCA binds to S1PR2, although the protein expressions of S1PR2 in human and mouse adrenal tissues have been confirmed in our data. Therefore, it is still possible that conjugated CDCA may activate S1PR2 through an indirect way.

As noted, TCDCA led to an induction of ERK phosphorylation (143) and TCDCA-induced steroidogenesis via ERK signaling pathway was weakened upon inhibition of S1PR2. This result is consistent with the previous study in rat hepatocytes using conjugated bile acids (65). Enhanced glucocorticoid synthesis and transcription abundance caused by conjugated CDCA diminished upon inhibition of ERK phosphorylation, which highlights the importance of ERK phosphorylation in the regulation of bile acids and links bile acid in adrenal gland to ERK and S1PR2.

SF-1 is a nuclear transcription factor, which is characterized to widely regulate the transcription of many genes engaged in steroidogenesis such as STAR, HSD3B2, CYP21 and MC2R (144, 145). As previously reported, SF-1 is a substrate of ERK.

Once phosphorylated by ERK, the transactivation activity of SF-1 will be significantly elevated (146). In this thesis, it is for the first time to report increased transactivation activity by TCDCA in H295R cells, at least to our knowledge. Application of an SF-1 inverse agonist attenuate steroidogenesis induced by TCDCA, linking conjugated bile acid and steroidogenesis successfully. Note that inhibition of ERK phosphorylation or S1PR2 led to reduced protein expression of SF-1. A previous study described PKA-mediated phosphorylation of SF-1 where inhibited PKA led to decreased SF-1 protein stability (147). Therefore, one can speculate that reduced protein expression of SF-1 in our research may be also due to lower protein stability as a result of inhibition of ERK phosphorylation or S1PR2. Hence our data show that inhibitions of different signaling knots of S1PR2-MEK-ERK-SF-1 pathway abolish effects of conjugated bile acids on adrenal glands, underlining the indispensability of this distinct pathway in the regulatory role of bile acids in glucocorticoid synthesis.

Despite differences in bile acid pool and differences in the regulation of bile acid transport and metabolism between mice and men (148, 149), murine cholestasis induced by CBDL and CDCA feeding showed several similarities to the H295R cell culture system treated with TCDCA in our hands. We observed induction of the key enzymes involved in glucocorticoid production as well as increased cortisol/corticosterone levels in both *in vivo* and *in vitro* models. Moreover, TCDCA also led to increased ERK phosphorylation in mouse adrenals, which seems to be a key mechanism in response to S1PR2 activation. Taken together, this evidence strongly supports the conclusion that bile acids directly regulate steroidogenesis.

One noteworthy result from animal experiments is that plasma ACTH levels were unchanged in CBDL and CDCA-fed mice while mRNA levels of MC2R were significantly increased by CBDL and CDCA feeding (Figure 7 A and B). The overexpression of MC2R, in addition to HPA axis, is probably due to elevated

transactivation activity of SF-1 as MC2R is reported to be upregulated by SF-1 (150). In addition, based on our TCDCA-challenged H295R cells incubated with serum-free media without ACTH, we also observed bile acid-induced cortisol secretion independent of HPA axis regulation. Therefore, bile acids may independently regulate transcription of MC2R and steroidogenic genes via S1PR2-MEK-ERK-SF-1 pathway, acting as a beneficial complement to the intrinsic HPA axis negative feedback regulation mechanism in adrenocortical tissue.

5.4 Prospect: potential clinical significance of our research

There might be potential clinical implications laying in our research for those with excessive circulating bile salt levels such as patients with jaundice, cholestasis, or liver cirrhosis. Based on our study, bile acids may not directly affect adrenal function under normal conditions. However, when it comes to diseases characterized by substantially increased serum bile acid levels, bile acids probably become an important regulator of adrenal gland and contribute to dysregulation of glucocorticoid balance. For example, there is a clinical and pathophysiologic link between adrenal function and liver disease for those with liver cirrhosis, which is known as hepato-adrenal syndrome. One plausible hypothesis is that the severe dysfunction of adrenal gland is related to elevated circulating bile acids, at least partially. Furthermore, supraphysiologically high bile acid level may also contribute to the development of osteoporosis which is a complication of long-term steroid therapy or hypercortisolemia (151), and frequently encountered in cholestatic diseases (152)

There are still some questions needing to be answered in future. We observed slight increase of phosphorylation of some PKA substrates. Intracellular cAMP levels were also mildly increased. However, it is still unclear how bile acid induced elevated PKA activity. Some GPCRs may be triggered through direct or indirect way by bile acids. In addition, since SF-1 also regulate transcription of MC2R and mRNA level of MC2R is increased dramatically in adrenals of CBDL and CDCA-feeding mice, it is

reasonable to hypothesize that under physiological conditions, up-regulated MC2R lead to stronger responsiveness to ACTH hence enhanced PKA activity via coupled G proteins. These need to be further clarified with more detailed experiments to verify. Previous literature also showed that FXR activated by bile acids induces GR expression (153), which may augment effects of elevated cortisol levels. Thus, role of FXR under cholestasis on adrenal gland is worthwhile being evaluated systematically. In addition, in patients with longstanding cholestasis, multiple mechanisms along HPA axis *in vivo* may counteract or interfere with the efficacy of bile acid on adrenocortical tissues. Whether the results observed in mouse models and cell culture assays still exist in human beings is needed to be answered in future.

To make a summary, in this thesis, for the first time, we proved that conjugated bile acids at pathophysiologically-relevant high concentrations can direct affect hormone synthesis in adrenocortical cells. Elevated corticosterone and cortisol synthesis in two different cholemic mouse models and *in vitro* cultured human adrenocortical cell line respectively were clearly observed. These results do not rely on intrinsic regulation mechanism of HPA axis or the classical bile acid receptors FXR and TGR5 but are dependent of the bile acid receptor S1PR2 and its downstream signaling target ERK and SF-1.

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