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# Bilirubin Reduces the Uptake of Estrogen Precursors and the Followed Synthesis of Estradiol in Human Placental Syncytiotrophoblasts via Inhibition and Downregulation of Organic Anion Transporter 4

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## ABSTRACT

**Estrogen biosynthesis in human placental trophoblasts requires the human organic anion transporter 4 (hOAT4)-mediated uptake of fetal derived precursors such as dehydroepiandrosterone-3-sulfate (DHEAS) and 16α-hydroxy-DHEA-S (16α-OH-DHEAS). Scant information is available concerning the contribution of fetal metabolites on the impact of placental estrogen precursor transport and the followed estrogen synthesis. This study substantiated the roles of bilirubin as well as bile acids (taurochenodeoxycholic acid, taurocholic acid, glycochenodeoxycholic acid, chenodeoxycholic acid) on the inhibition of hOAT4-mediated uptake of probe substrate 6-carboxylfluorescein and DHEAS in stably transfected hOAT4-Chinese hamster ovary cells, with the $IC_{50}$ of 1.53 and 0.98 μM on 6-carboxylfluorescein and DHEAS, respectively, for bilirubin, and 90.2, 129, 16.4, and 12.3 μM on 6-CF for taurochenodeoxycholic acid, glycochenodeoxycholic acid, taurocholic acid, and chenodeoxycholic acid. Bilirubin (2.5–10 μM) concentration-dependently inhibited the accumulation of estradiol precursor DHEAS in human choriocarcinoma JEG-3 cells (reduced by 60% at 10 μM) and primary human trophoblast cells (reduced by 80% at 10 μM). Further study confirmed that bilirubin (0.625–2.5 μM) concentration-dependently reduced the synthesis and secretion of estradiol in primary human trophoblast cells, among which 2.5 μM of bilirubin reduced the synthesis of estradiol by 30% and secretion by 35%. In addition, immunostaining and Western blot results revealed a distinct downregulation of hOAT4 protein expression in primary human trophoblast cells pretreated with 2.5 μM of bilirubin. In conclusion, this study demonstrated that bilirubin reduced the uptake of estrogen precursors and the followed synthesis of estradiol in human placenta *via* inhibition and downregulation of organic anion transporter 4.**

## SIGNIFICANCE STATEMENT

**Fetal metabolites, especially bilirubin, were first identified with significant inhibitory effects on the hOAT4-mediated uptake of estrogen precursor DHEAS in hOAT4-CHO, JEG-3 and PHTCs. Bilirubin concentration-dependently suppressed the estradiol synthesis and secretion in PHTCs treated with DHEAS, which was synchronized with the decline of hOAT4 protein expression. Additionally, those identified bile acids exhibited a weaker inhibitory effect on the secretion of estradiol.**

## Introduction

Human placental trophoblasts depend largely on the supply of external precursors, such as dehydroepiandrosterone-3-sulfate (DHEAS) and 16α-hydroxy-DHEAS (16α-OH-DHEAS) for further synthesis of estrogens with advancing gestation (Morel et al., 2016). It has been shown that both fetal and maternal precursor DHEAS are used in about equal proportions in the biosynthesis of estrone (E1) and estradiol (E2), while 90% of the fetal-derived 16α-OH-DHEAS for the formation of estriol (E3), which increases drastically with fetal development, are of fetal origin (Pasqualini, 2005). Serving as the main source of maternal estrogens, the placenta exquisitely expresses organic anion transporter 4 (OAT4) and organic anion transporting polypeptide 2B1 (OATP2B1, former name OATP-B), which are closely involved in the placental uptake of fetal derived hydrophilic steroid sulfates in cytotrophoblast membranes and at the basal surface of the syncytiotrophoblast (Schweigmann et al., 2014; Tomi et al., 2015), permitting temporary but significant biologic functions during gestation. Former studies have ascertained that OAT4 contributes to the transport of DHEAS and exclusively mediates the uptake of 16α-OH-DHEAS (Schweigmann et al., 2014; Tomi et al., 2015).

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**ABBREVIATIONS:** BLB, bilirubin; CA, cholic acid; CDCA, chenodeoxycholic acid; 6-CF, 6-carboxylfluorescein; CHO, Chinese hamster ovary; DHEAS, dehydroepiandrosterone-3-sulfate; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceradehyde-3-phosphate dehydrogenase; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GAPDH, glyceradehyde-3-phosphate dehydrogenase; GDCA, glycodeoxycholic acid; hOAT4, human organic anion transporter 4; HSA, human serum albumin; IMA, ischemia-modified albumin; OAT, organic anion transporter; OATP2B1, organic anion transporting polypeptide 2B1; 16α-OH-DHEAS, 16α-hydroxy-DHEAS; PHTC, primary human trophoblast cell; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid..

Fetal-derived potentially toxic cholephilic organic anions, such as biliary pigments and bile acids cannot be disposed by the immature fetal liver, thus the placenta and the maternal liver play a key role in the metabolism and excretion of those metabolic waste materials. Due to the active heme catabolism and the following high rate of bilirubin production, together with a low expression of bilirubin uridine diphosphate-glucuronosyl transferase in the fetal liver, unconjugated bilirubin, mostly binding to serum albumin or $\alpha$-fetoprotein (Aoyagi et al., 1979), has higher concentrations in fetal than in maternal serum (Macias et al., 2009). Fetal-derived bile acids from the meconium and the gallbladder have been identified in several studies (Setchell et al., 1988; Naritaka et al., 2015) using liquid chromatography-electrospray ionization-tandem mass spectrometry, which enabled us to elucidate the developmental process of fetal bile acid metabolism reflecting fetal physiologic conditions. Unlike human adults, fetal bile acids are mainly conjugated with taurine, followed by glycine (McIlvride et al., 2017). Fetal heterogeneous bile acids detected in gallbladder bile and the intestinal contents mostly consist of taurocholic acid (TCA) and taurochenodeoxycholic acid (TCDCA), to the extent of about 80 to 90%, with the remainder consisting of glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA). However, it remains uncertain whether those fetal metabolites have impact on the activity and expression of placental hOAT4-mediated uptake of estrogen precursors.

Many studies have indicated that a marked decrease in estrogen, including E1, E2, and E3, together with DHEAS levels may be closely related to the impact of multiple pathologies of the pregnancy, among which intrahepatic cholestasis (Leslie, et al., 2000; Troisi et al., 2003; Wang et al., 2011; Pařízek et al., 2016) and preeclampsia (Acikgoz S, et al., 2013; Berkane et al., 2017) have been constantly arousing people's interest and been extensively investigated, yet no consensus has been reached. Scant information is available concerning the contribution of fetal metabolites on the impact of placental estrogen precursor transporters and the further estrogen synthesis. More detailed information with respect to hOAT4 and its roles during physiologic and pathologic gestational conditions are to be illustrated.

With those in mind, the purpose of the present study is to substantiate the roles of fetal metabolites, including bilirubin and bile acids, on the inhibition of hOAT4-mediated-uptake of estrogen precursors into placental syncytiotrophoblasts from fetus, and to examine the contribution of fetal metabolites on estradiol synthesis and hOAT4 protein expression at the cellular level, using human choriocarcinoma JEG-3 cells and primary human trophoblast cells (PHTCs).

## Materials and Methods

**Materials.** Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), Dulbecco's modified Eagle's medium/F12 medium were obtained from GIBCO (Invitrogen Life Technologies, USA). 6-carboxylfluorescein (6-CF), human serum albumin (HSA) were purchased from Sigma-Aldrich (St. Louis, MO). Cholic acid (CA), TCA, TCDCA, GCA, GCDCA, chenodeoxycholic acid (CDCA), GDCA, and TDCA were provided by Aladdin Co., Ltd. (Shanghai, China). Bilirubin (BLB) was obtained from Macklin Biochemical Co., Ltd. (Shanghai, China). DHEAS was purchased from Meilun Biologic Co., Ltd. (Dalian, China). Acetonitrile was obtained from Tedia (Fairfield, TX). Bicinchoninic acid protein assay kit was purchased from Beyotime Institute of Biotechnology (Beyotime, China). SDS was obtained from Amresco (Solon, OH). Anti-SLC22A11/OAT4 antibody (ab76385) and G418 were obtained from Abcam (Cambridge, MA). Anti-HSD17$\beta$1 antibody (db4038) and anti-Aromatase antibody (db3890) were provided by Diagbio Co., Ltd. (Hangzhou, China). Glyceradehyde-3-phosphate dehydrogenase (GAPDH) antibody, and the anti-mouse and anti-rabbit secondary antibodies were purchased from Multi Sciences (Lianke) Biotech Co., Ltd. (Hangzhou, China). Other chemicals or solvents were of the highest grade commercially available.

Blank vector (pEnter), and hOAT4 (SLC22A11) expression plasmid were purchased from ViGene Biosciences, Inc. (Shandong, China.)

**Cell Culture.** Human choriocarcinoma JEG-3 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Chinese hamster ovary (CHO) cells were kindly provided by Prof. Shuqing Chen, College of Pharmaceutical Sciences, Zhejiang University. JEG-3 and CHO cells were cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified air/$CO_2$ incubator (5% v/v).

**Establishment of Stably Transfected hOAT4-CHO cells.** Recombinant hOAT4-pc3.1 plasmid was constructed successfully from hOAT4-pEnter plasmid purchased from ViGene Biosciences, Inc. before CHO cells were seeded in 6-well plates at appropriate density. On day 2, CHO cells were transiently transfected with hOAT4-pc3.1 recombinant plasmid or blank vector (mock) using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, USA) based on the manufacturer's protocol when they reached 60 to 70% confluence. On the following 14 days, the cultured medium of CHO cells transfected with hOAT4-pc3.1 plasmid was replaced every day with the existence of high concentrations of G418 (approx. 900 micrograms/ml). The hOAT4-CHO cells remained were seeded at an exquisite density of 1/96 well (1/200 $\mu$l cultured medium). On day 3 after seeding, the stably transfected hOAT4-cell wells were marked, and their functions were further validated by comparing the accumulation results of probe substrates in stably-expressed cells with that in mock cells.

**Cellular Accumulation.** The cellular accumulations of 6-CF/DHEAS in JEG-3, hOAT4-CHO cells and primary human trophoblast cells (PHTCs) were performed as the method described in our previous study (Bai et al., 2017; Ma et al., 2017). Briefly, the cells were pre-incubated with MES (NaCl, 140 mM; D-glucose, 5.6 mM; KCl, 5.4 mM; $MgSO_4{\cdot}7H_2O$, 0.8 mM; $KH_2PO_4$, 0.4 mM; $CaCl_2$, 1.3 mM; $NaHCO_3$, 4.2 mM; $Na_2HPO_4{\cdot}12H_2O$, 0.2 mM;2-morpholinoethanesulfonic acid, 10 mM; pH 6.0) buffer at 37°C for 20 minutes with or without inhibitors, and then MES buffer containing 6-CF (5 $\mu$M)/DHEAS (10 $\mu$M) in the absence or presence of inhibitors was added to initiate the accumulation process. This procedure was terminated by adding ice-cold PBS solution quickly at the designated time and removing the incubation buffer. Then the remained bottom cells were washed three times with ice-cold PBS before they were lysed with 100 $\mu$M of 0.1% sodium dodecyl sulfate.

All experiments were performed in triplicate for at least three separate experiments. The concentrations of DHEAS, MTX, BLB, TCDCA, TCA, GCDCA, and CDCA in the cells were quantified with liquid chromatography tandem mass spectrometry and then normalized to the total protein content detected with BCA assay in the lysates. The concentrations of 6-CF ($\lambda_{ex}$490 nm, $\lambda_{em}$525 nm) were determined by a microplate reader (Spectra Max M2, Molecular Devices, USA). The accumulation results in the presence of inhibitors were expressed as the percentage of the vehicle group (fold of control).

**Inhibitory Effects of Fetal Metabolites on hOAT4 in PHTCs.** Primary human trophoblast cells were isolated from human uncomplicated placenta delivered at term (38–40 weeks) as the method reported previously with minor modifications (Bai et al., 2017; Ma et al., 2017; Zeng et al., 2019). Briefly, aliquots of villous from the maternal surface of the placenta were cut away from vessels and washed with PBS containing 1% penicillin–streptomycin 8–9 times. Five hundred milliliters of DMEM containing 25 mM of glucose (high-glucose DMEM), 0.07% trypsin, and 0.2 mg/ml DNase I (Sigma) were prepared before the digestion periods. Then, the tissue was minced and transferred to 200 ml of the DMEM and incubated in a shaking water bath at 37°C for four different periods of time (30, 30, 15, and 15 minutes). The third and fourth digestions were mixed and filtered through a nylon mesh, and the pellets were collected after being centrifuged at 2500g for 10 minutes at 4°C. The cells were resuspended in 10 ml of DMEM medium containing 10% FBS, which was layered over a 5–65% Percoll (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient at stepwise increments of 5% and centrifuged at 2500g for 20 minutes at 4°C. The cytotrophoblasts in the middle layer were collected and planted at $1.5\times10^6$ cells per well in 12-well plates for culture in DMEM containing 10% FBS. The accumulation assay was performed with the method for JEG cells at 24 hours after seeding.

**Enzyme-Linked Immunosorbent Assays.** Estradiol in the conditioned medium was quantified using a human estradiol enzyme immunoassay kit (Yifeixue Biotechnology, Nanjing, China) based on the manufacturer's protocol. Cells were cultured in 5 $\mu$M of DHEAS with or without the presence of bile acids and bilirubin for 24 hours before the later determination of estradiol secretion. Estradiol concentrations in the conditioned medium and the bottom cells were determined by means of an enzyme-linked immunosorbent assay. The bottom cells in the 12-well plate were dissolved in 150 $\mu$l of 0.1% SDS, and then protein content was measured by means of bicinchoninic acid assay to normalize the results.

**Immunostaining of hOAT4.** The isolated cytotrophoblasts were treated for 24 hours with cultured medium containing DMSO and 2.5 $\mu$M of DMSO 2 hours after the seeding. The bottom cells were washed by PBS three times and placed in fresh 4% neutral-buffered paraformaldehyde before it was probed with antibody against hOAT4 (Abcam, Cambridge, MA), followed by staining with CoraLite 488-conjugated goat anti-rabbit antibodies (Proteintech, Wuhan, China). 4',6-diamidino-2-phenylindole was then used to identify nuclei. Green labeling indicates hOAT4-positive cells (×200).

**Western Blot Analysis.** Western blot was performed following standard protocols: cells were harvested and lysed using radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China). Protein extracts were subjected to the further SDS-PAGE analysis and subsequently transferred to a polyvinylidene difluoride membrane (0.45 $\mu$M, Millipore, MA, USA). The membranes were blocked with 5% non-fat in tris-buffered saline with Tween followed by antibody hybridization and then visualized in a Western blotting detection system (LI-COR Biosciences, Lincoln, NE).

**RNA Isolation, cDNA Synthesis, and Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) Assays.** Total RNAs were isolated using RNA simple Total RNA Kit (Tiangen, China) before the cDNAs were synthesized using PrimeScript RT reagent Kit (Takara Bio, Tokyo). Then, a real-time PCR procedure was performed using SYBR Premix Ex TaqTM II (Takara Bio). Expression of the target mRNAs were normalized to the house-keeping gene GAPDH.

**Liquid Chromatography Tandem Mass Spectrometry Quantifications of Fetal Metabolites and DHEAS.** The concentrations of DHEAS and bilirubin in the samples were quantified by an Agilent 1290/6460 liquid chromatography mass spectrometer with a triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, USA). For DHEAS determination, 40 $\mu$l of the cell lysate was mixed with 200 $\mu$l of acetonitrile containing the internal standard (100 nM diclofenac) for 5 minutes before the mixture was centrifuged at 16,000g for 15 minutes. The supernatant was further analyzed by liquid chromatography tandem mass spectrometry. Isocratic chromatographic separation was performed on a Porshell $C_{18}$ column (2.7 µm, 2.1 × 50 mm) at 30°C with a gradient elution (0–0.9 minutes, 90% of A; 0.9-1.1 minutes, 90–10% of A; 1.1-3.0 minutes, 10% of A; 3.0–3.2 minutes, 10–90% of A; 3.2–5.0 minutes, 90% A), at 0.25 ml/min, where mobile phase A and B were water containing 0.1% formic acid and acetonitrile 0.1% formic acid, respectively. An electrospray ionization source was used to conduct mass spectrometric analysis in negative ion mode. Quantification was obtained using multiple reaction monitoring mode at m/z transitions of 367.1 > 367.1 for DHEAS and 295.9 > 251.8 for diclofenac. Fragmentor voltage was set at 190 and 75 V, and collision energy was 5 and 7 V for DHEAS and diclofenac, respectively.

To ascertain if those fetal metabolites were substrates of hOAT4, cellular accumulation of bilirubin/TCDCA/TCA/GCDCA/CDCA samples were measured using the sample preparation method described for the cells mentioned above. Isocratic chromatographic separation for bilirubin was performed on a Porshell $C_{18}$ column (2.7 µm, 2.1 × 50 mm) at 30°C with a gradient elution (0–0.9 minutes, 90% of A; 0.9–1.1 minutes, 90–10% of A; 1.1–3.0 minutes, 10% of A; 3.0–3.2 minutes, 10–90% of A; 3.2–5.0 minutes, 90% A), at 0.25 ml/min, where mobile phase A and B were water and acetonitrile, respectively. Isocratic chromatographic separation for bile acids was performed on a Porshell $C_{18}$ column (2.7 µm, 2.1 × 50 mm) at 30°C with a gradient elution (0–1.5 minutes, 80% of A; 1.5–2.0 minutes, 80–40% of A; 2.0–3.0 minutes, 40% of A; 3.0–5.0 minutes, 40–80% of A; 3.2–5.0 minutes, 80% A), at 0.25 ml/min, where mobile phase A and B were water and acetonitrile, respectively. A negative-ion-mode ESI source was used for mass spectrometric analysis. Quantifications were obtained at m/z transitions of 295.9 > 251.8 for diclofenac (internal standard for bilirubin), 583.3 > 285 for bilirubin, 407 > 343 for CA (internal standard for bile acids), 514 > 124 for TCA, 498 > 124 for TCDCA, 391.2 > 391.2 for CDCA, and 448.3 > 74 for GCDCA. Fragmentor voltage was set at 75, 150, 280, 150, 275, 250, and 200 V, and collision energy was 7, 22, 10, 10, 10, 0, and 35V for diclofenac, bilirubin, CA, TCA, TCDCA, CDCA, and GCDCA, respectively.

The methods were validated according to United States Food and Drug Administration guidelines and satisfactory specificity, precision (inter- and intra-assay), accuracy (inter- and intra-assay), and matrix effect were demonstrated.

**Data Analysis.** Data are expressed as mean ± SD. *In-vitro* experiments were conducted at least three times in triplicate. Unpaired Student's *t* test was performed between two groups, and one-way analysis of variance followed with Dunnett's or Tukey's post hoc test was applied for more than two groups through the GraphPad Prism version 8.0 analysis. $P$ values $< 0.05$ were considered statistically significant.

## Results

**Fetal Metabolites Were Identified to Be the Inhibitors of hOAT4.** OAT4 is one of the most abundantly expressed SLC transporters in human placenta facing the fetal side and plays important roles in the uptake of fetal-derived DHEAS and 16α-OH-DHEAS for the following estrogen synthesis. Here, we investigated whether those fetal metabolites, such as bilirubin and bile acids, have inhibitory effects on OAT4 activity. As shown in Fig. 1, bilirubin (BLB), whose metabolism and excretion in the fetus is much less efficient than that in the adult due to the immaturity of fetal liver, had fascinatingly intense inhibitory effects on the accumulation of 6-CF (a known substrate of hOAT4) in stably transfected hOAT4-CHO cells with the $IC_{50}$ value reaching up to 1.53 $\mu$M (Fig. 1a). Additionally, bile acids in the fetal compartment, including TCDCA, GCDCA, TCA, and CDCA reduced the hOAT4-mediated uptake of 6-CF, with the $IC_{50}$ values of 90.2, 129, 16.4, and 12.3 $\mu$M, respectively (Fig. 1b-e). Other tested bile acids, such as GCA, CA, TDCA, and GDCA may not impact the transport activity of hOAT4 (Fig. 1f). Despite the inhibitory roles of those fetal metabolites confirmed above, they are not substrates of hOAT4 based on their accumulation data in mock and hOAT4-CHO cells with or without the existence of probenecid (100 $\mu$M), an identified OAT4 inhibitor (Fig. 1g). Additionally, bilirubin seems not to impact other OAT transporters, including OAT1, OAT2, and OAT3 (Fig. 1i-k); thus, bilirubin is potentially a specific inhibitor of OAT4.

**Fetal Metabolites Inhibited hOAT4-Mediated Uptake of Estrogen Precursors.** Since hormones derived from the placenta play a critical role in establishment and subsequent progression of human pregnancy, it dramatically aroused our interest to investigate whether those metabolites play roles in the hOAT4-mediated transport of estrogen precursors and subsequently reducing the estrogen synthesis. Considering that unconjugated bilirubin generally binds with albumin, to examine the inhibitory effects on hOAT4-mediated uptake of substrates in the mimic real fetal compartment, we conducted the accumulation procedure of 6-CF and estrogen precursor DHEAS at varied molar ratios of bilirubin/albumin (Ahlfors and Wennberg, 2004; Calligaris et al., 2007; Morioka et al., 2015) with the presence of 10 $\mu$M (Fig. 2) of HSA in the accumulation buffer.

As shown in this section, the bilirubin-albumin system containing bilirubin with concentrations ranging from 0.625 to 10 $\mu$M in the presence of 10 $\mu$M HSA buffer performed significant inhibitory effects on the accumulation of 6-CF (reduced by 60%, at 10 $\mu$M, $P < 0.001$, Fig. 2a) and estrogen precursor DHEAS (reduced by 80%, at 10 $\mu$M, $P < 0.001$, Fig. 2b) in hOAT4-CHO cells with the $IC_{50}$ value of 0.98 $\mu$M for DHEAS, but exerted little inhibitory effects on OATP2B1 (**Fig. S2**).

Moreover, bile acids, including TCDCA, GCDCA, CDCA, and TCA showed relatively weaker yet concentration-dependent inhibitory effects on the accumulation of DHEAS in hOAT4-CHO cells ($P < 0.05$, Fig. 2c).

**Fetal Metabolites Inhibited the Accumulation of DHEAS in JEG-3 and PHTCs.** To further confirm the roles of fetal metabolites on hOAT4-mediated transport through placenta, we measured the inhibitory effects of bilirubin on the accumulation of DHEAS in JEG-3, a

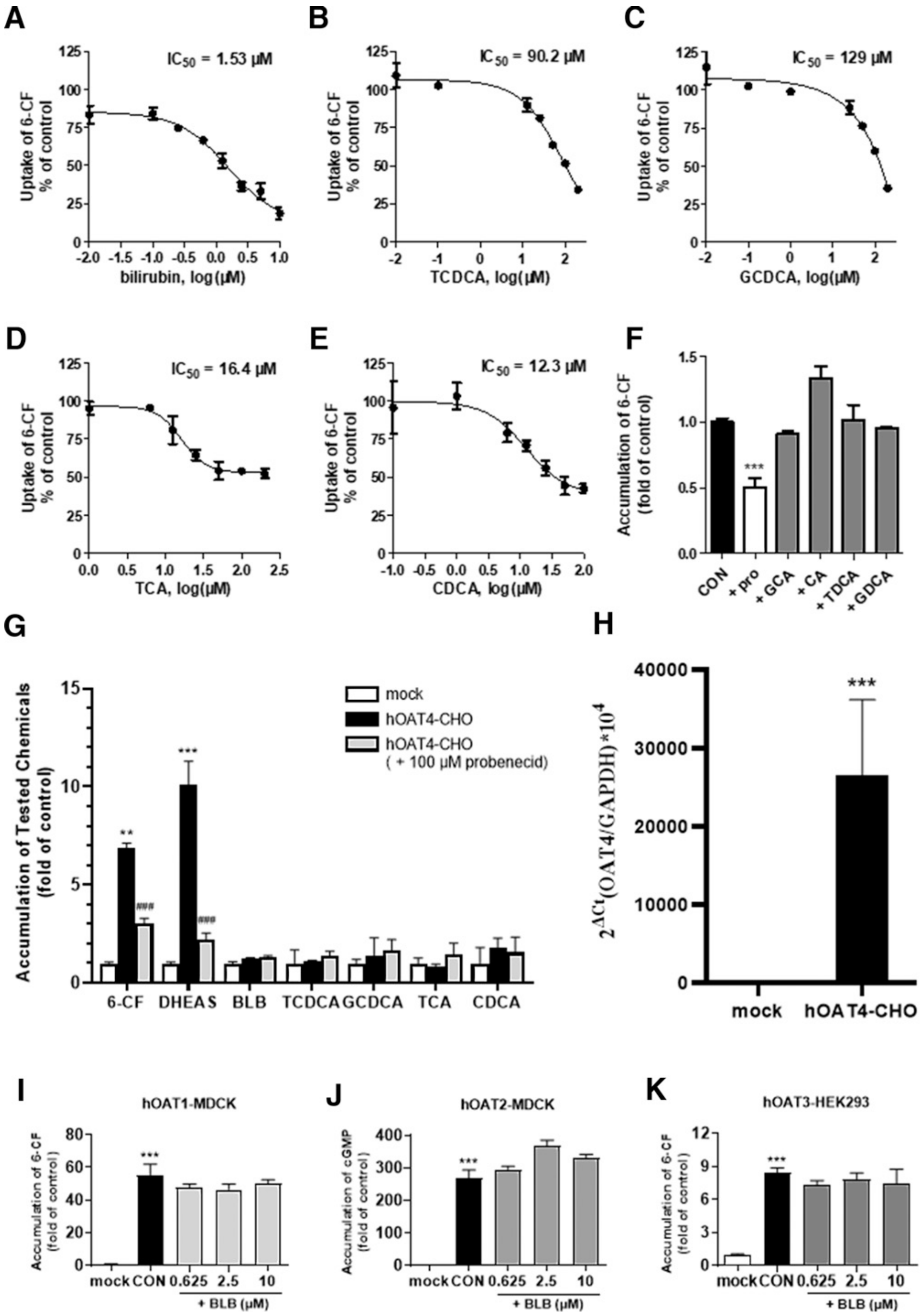


**Fig. 1.** Identification of inhibitors and substrates of organic anion transporter 4 (OAT4) from fetal metabolites, including (A) Bilirubin, (B) taurochenodeoxycholic acid, (C) glycochenodeoxycholic acid, (D) taurocholic acid, and (E) chenodeoxycholic acid. (F) Tested bile acids that are not inhibitors of hOAT4 were listed. The accumulation was expressed as percent or fold of 6-carboxylfluorescein (6-CF, 5 $\mu$M) without the fetal metabolites in human OAT4-Chinese hamster ovary (hOAT4-CHO) cells. Compared with the hOAT4-CHO cells without probenecid (CON), $^{***}P < 0.001$. (g) The accumulations of fetal metabolites (50 $\mu$M) in mock and hOAT4-CHO cells. The accumulation was expressed as fold of 6-CF (5 $\mu$M), DHEAS (10 $\mu$M) or tested fetal metabolites in mock cells without probenecid (100 $\mu$M). (h) Quantitative real-time polymerase chain reaction was carried out to identify the expression of hOAT4 mRNA in hOAT4-CHO cells. (i-k) Identifications of the bilirubin inhibitory effects on organic anion transporter 1-Mardin-Darby canine kidney, organic anion transporter 2-Mardin-Darby canine kidney, and organic anion transporter 3 human embryonic kidney stably transfected cells constructed and preserved in our laboratory. Functions of tested OAT1-3 cells have been previously identified by their probe substrates (5 $\mu$M of 6-CF, 10 $\mu$M of cyclic guanosine monophosphate, and 5 $\mu$M of 6-CF, respectively). Compared with the mock cells, $^{***}P < 0.001$; compared with the hOAT4-CHO cells without probenecid, $^{\#\#\#}P < 0.001$. Data were expressed as mean ± S.D., n = 3, from three independent experiments conducted in triplicate.

well-characterized immortalized human trophoblast choriocarcinoma cell line. As shown in Fig. 3a, bilirubin potently reduced the accumulation of DHEAS in the presence of 10 $\mu$M HSA in JEG-3 in a concentration-dependent manner (reduced by 60%, at 10 $\mu$M, $P < 0.001$). PHTCs (primary human trophoblast cells) were used to further assess the inhibitory roles of those metabolites. The results revealed that the bilirubin-albumin system containing 10 $\mu$M of HSA exhibited obviously concentration-dependent inhibitory effects on the accumulation of the

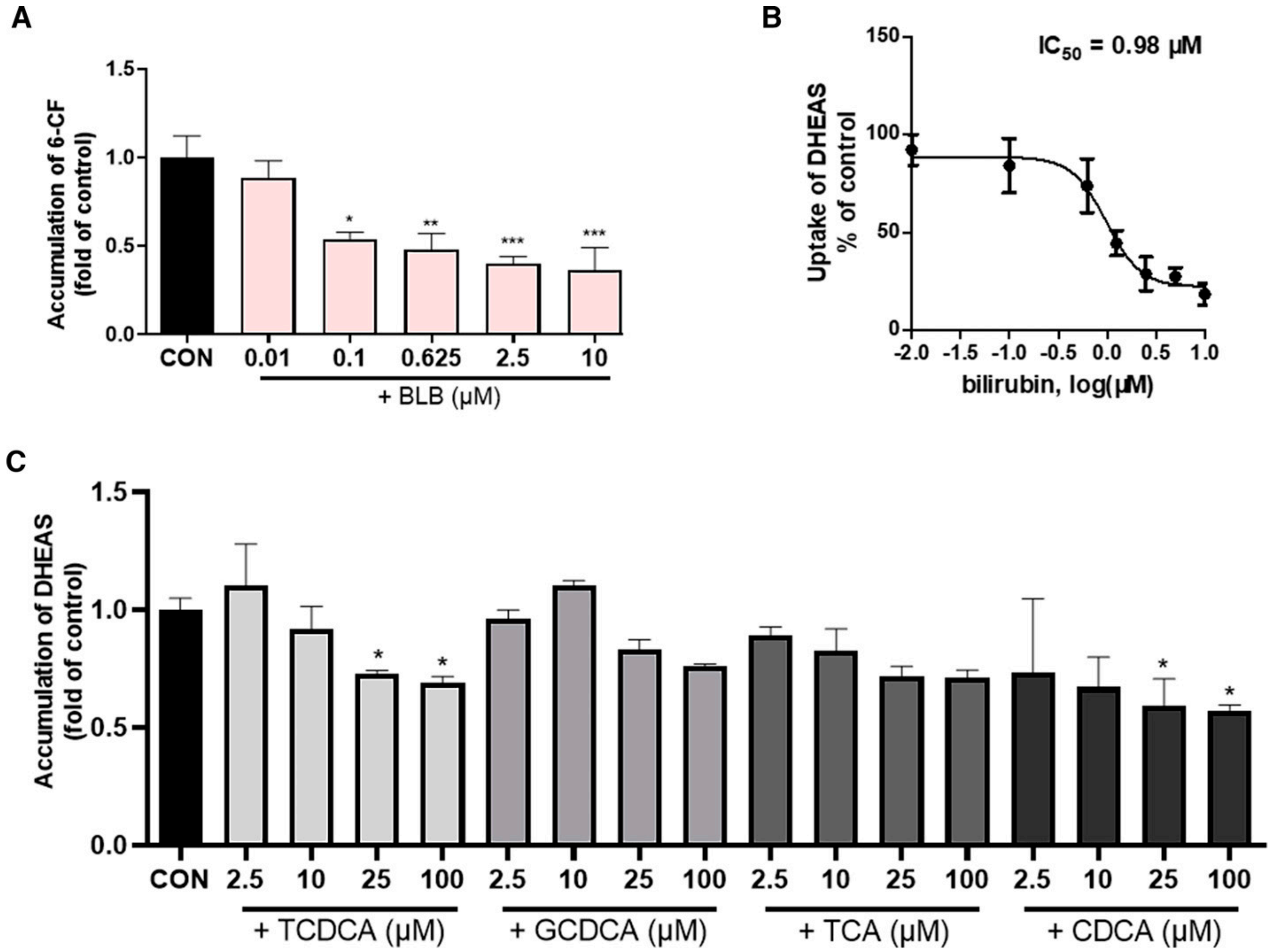


**Fig. 2.** The inhibitory effects of fetal metabolites on the uptake of 6-carboxylfluorescein (5 $\mu$M) or estrogen precursor dehydroepiandrosterone-3-sulfate (10 $\mu$M) in human organic anion transporter 4-Chinese hamster ovary (hOAT4-CHO) cells. The bilirubin-albumin system performed inhibitory effects on the accumulation of (A) 6-carboxylfluorescein and (B) dehydroepiandrosterone-3-sulfate in hOAT4-CHO cells in the presence of 10 $\mu$M of human serum albumin. Compared with the substrate uptake in hOAT4-CHO cells without inhibitors (CON), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (C) Bile acids performed weaker inhibitory effects on the uptake of dehydroepiandrosterone-3-sulfate in hOAT4-CHO cells. Compared with the uptake in hOAT4-CHO cells without bilirubin or bile acids (CON), $*P < 0.05$. The accumulation was expressed as fold of CON. All cells were incubated at 37°C for 3 minutes. Data were expressed as mean ± S.D., n = 3, from three independent experiments conducted in triplicate.

estrogen precursor DHEAS in PHTCs (reduced by 80%, bilirubin at 10 $\mu$M, $P < 0.001$, Fig. 3b).

Additionally, fetal bile acids (100 $\mu$M), including TCDCA, GCDCA, TCA, and CDCA did exhibit a certain impact on the accumulation of DHEAS in PHTCs (Fig. 3d).

**Bilirubin Reduced the Estradiol Synthesis and Secretion from PHTCs.** The PHTCs were further used to ascertain estradiol secretion inhibition roles of those metabolites mentioned above in placenta, as illustrated in Fig. 4. Cells were pre-cultured with 5 $\mu$M of DHEAS with or without bilirubin or bile acids for 24 hours before the later determination of estradiol secretion. As shown in Fig. 4, estradiol concentrations in the medium (Fig. 4a) and cells (Fig. 4b) were concentration-dependently inhibited by bilirubin pretreatment, among which 2.5 $\mu$M of bilirubin reduced the synthesis of estradiol by 30% and secretion by 35%. We further confirmed that bile acids (50 $\mu$M) showed marginal inhibitory effects on the secretion of estradiol in PHTCs ($P < 0.05$, Fig. 4c).

**Bilirubin Downregulated the hOAT4 Protein Expression in PHTCs.** To further explore whether bilirubin performed downregulation roles in such an E2-suppressing procedure, the cells were carefully collected for further determinations of hOAT4 protein expression through immunostaining and Western blot after pretreatment with bilirubin ranging from 0.625 to 2.5 $\mu$M for 24 hours. Bilirubin reduced the immune fluorescence intensity of hOAT4 (Fig. 5a) and inhibited hOAT4 protein expression in a concentration-dependent manner (Fig. 5b). The protein and mRNA expressions of those steroidogenic enzymes involved in the synthesis and metabolism of estradiol were determined and of little difference (Fig. 5b-c), indicating that they contributed little to the lowering of estradiol secretion in PHTCs. The hOAT4 protein expression in PHTCs pretreated with bilirubin were downregulated, while the mRNA expression (Fig. 5c) seemed not to be altered, suggesting that post-translational modifications may be involved in this process.

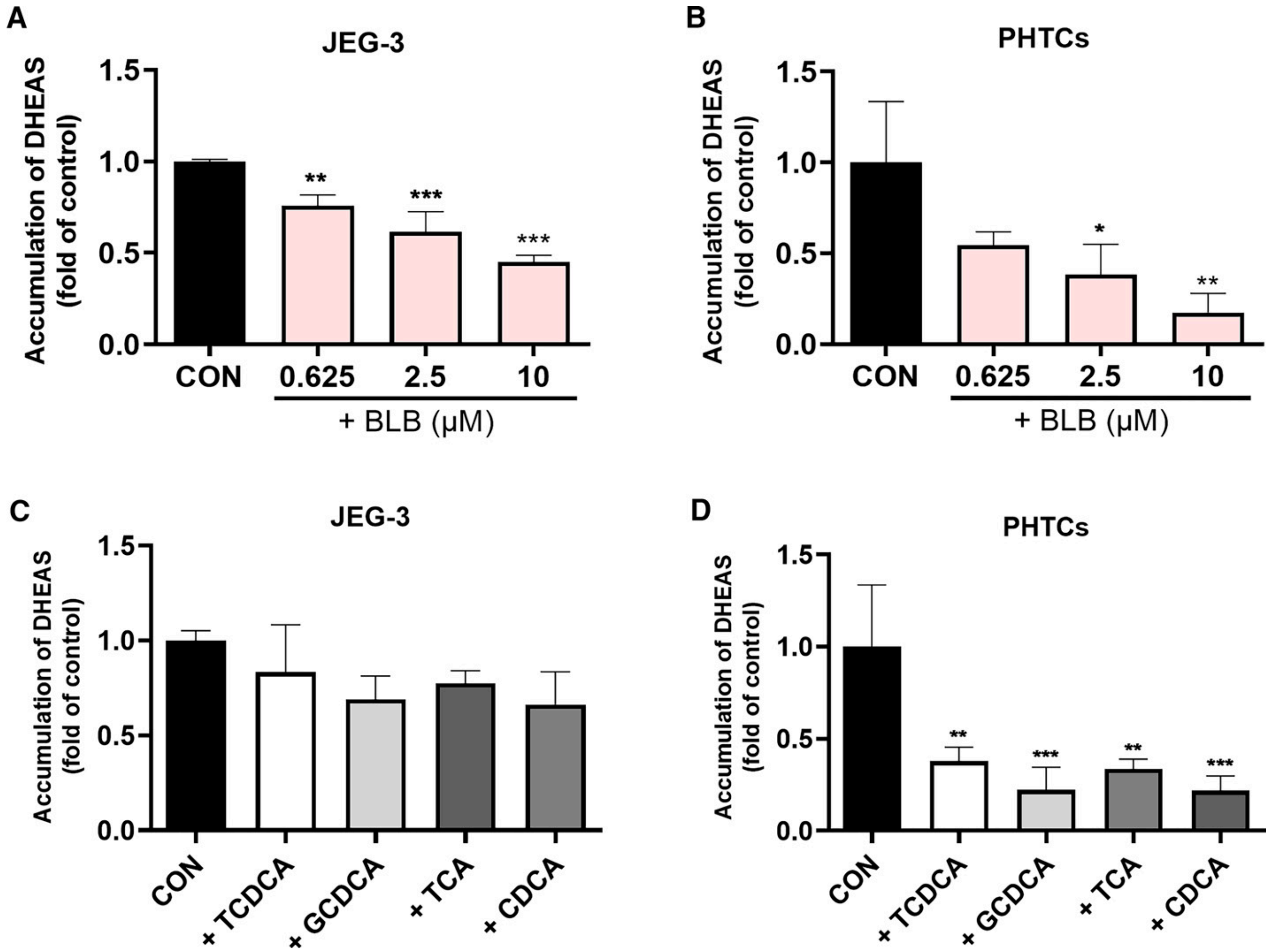


**Fig. 3.** Inhibitory effects on hOAT4-mediated uptake of dehydroepiandrosterone-3-sulfate (10 $\mu$M) in JEG-3 cell lines and primary human trophoblast cells by bilirubin and bile acids. Bilirubin with different concentrations in the presence of 10 $\mu$M human serum albumin showed inhibitory effects on dehydroepiandrosterone-3-sulfate accumulation in JEG-3 (A) and primary human trophoblast cells (B). Bile acids including taurochenodeoxycholic acid, glycochenodeoxycholic acid, taurocholic acid and chenodeoxycholic acid (100 $\mu$M) performed relatively weaker inhibitory effects on the accumulation of dehydroepiandrosterone-3-sulfate in JEG-3 (C) and primary human trophoblast cells (D). Compared with the accumulation without bilirubin or bile acids (CON) . *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. All cells were incubated at 37°C for 3 minutes. The accumulation was expressed as fold of CON. Data were expressed as mean ± S.D., n = 3, from three independent experiments conducted in triplicate.

## Discussion

This study gave solid evidence that bilirubin in the fetal compartment exhibited significant inhibitory effects on hOAT4-mediated estrogen precursor DHEAS transport in hOAT4-CHO cells, JEG-3 and PHTCs. Bilirubin concentration-dependently suppressed the estradiol synthesis and secretion in PHTCs, which was synchronized with the decline of hOAT4 protein expression. Additionally, those identified bile acids rendered a weaker inhibitory tendency in transporting hOAT4-mediated substrates.

The hOAT4-mediated uptake of DHEAS (Cha et al., 2000; Ugele et al., 2008) and 16α-OH-DHEAS (Schweigmann et al., 2014; Tomi et al., 2015) showed saturable kinetics and followed the Michaelis-Menten equation with nonlinear regression analysis yielding $K_m$ values of 29.2 ± 3.4 and 7.35 ± 3.5 $\mu$M and $V_{max}$ values of 620 ± 71 and 85.5 ± 22.2 pmol/mg protein/min. DHEAS is transported by hOAT4 and hOATP2B1 for further metabolism to estrone (E1) and estradiol (E2), while 16α-OH-DHEAS accumulation is only mediated by hOAT4 to be further transformed to estriol (E3). Although hOATP2B1 is highly expressed in PHTCs and placenta, the affinity of DHEAS toward OATP2B1 ($K_m$ of 210.8 $\mu$M, $V_{max}$ of 602 pmol/mg protein/min) was about 10 times lower than that in hOAT4. Thus, we assumed that hOAT4 plays an essential role in the accumulation of estrogen precursors during pregnancy and the inhibitory effects of fetal metabolites on hOAT4-mediated substrate uptake provide a novel insight into the correlation between abnormal fetal derived waste with maternal estrogen levels.

Orthologs of OAT4 are found only in humans, but not in rodents (Cha et al., 2000), which makes it impossible to conduct animal-based studies for further illuminations of hOAT4. Total bilirubin concentration in fetal blood ranged from 3.2 to 19.5 $\mu$M (Nava et al., 1996; Sikkel et al., 2004), in which case the conjugated bilirubin concentration was less than 10% of the total bilirubin concentration. As illustrated before, fetal bilirubin is mostly composed of unconjugated bilirubin binding to serum albumin or α-fetoprotein, and plasma unbound free bilirubin levels at any given total bilirubin or bilirubin/albumin ratio can vary widely due to varying concentrations of albumin. At a given total bilirubin, 30 $\mu$M of HSA and 10% (vol/vol) FBS yielded comparable free bilirubin values (Sebastian D. C., 2007). The bilirubin-albumin system containing 0.01 to 10 $\mu$M of bilirubin in the presence of 10 $\mu$M of HSA buffer

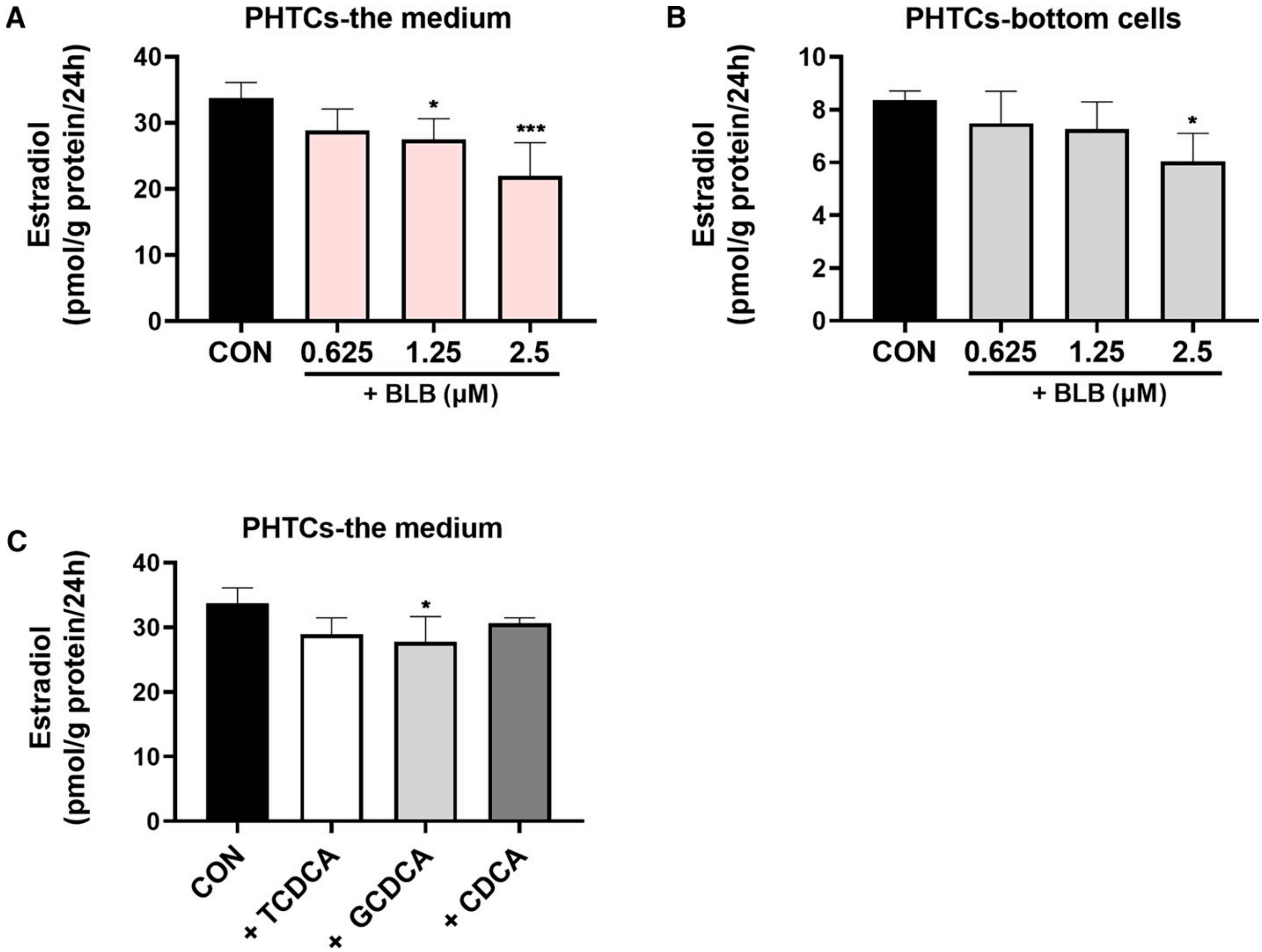


**Fig. 4.** Bilirubin or bile acids reduced estradiol concentration in cultural medium (A, C) and primary human trophoblast cells (B). Cells were cultured with dehydroepiandrosterone-3-sulfate (5 $\mu$M) in the absence or presence of bilirubin/bile acids (50 $\mu$M) at 37°C for 24 hours. Estradiol concentrations in the medium cells were determined by means of an enzyme-linked immunosorbent assay. Compared with the control group without bilirubin or bile acids (CON), $^{*}P < 0.05$, $^{***}P < 0.001$. Data were expressed as mean ± S.D., n = 3, from three independent experiments conducted in triplicate.

revealed evident concentration-dependent inhibitory effects on the accumulation of 6-CF as well as estrogen precursor DHEAS (Fig. 2-3) in hOAT4-CHO cells while exhibiting no influence on OATP2B1 (Fig. S2). Mean fetal total bile acid concentration reached 3.6 $\mu$M (range 3.1-4.1) (Estiu et al., 2015) and 2.2 $\mu$M (range 1.8-2.9) (Vasavan et al., 2021). TCDCA and TCA make up 80–90% of total fetal bile acid profile, and bile acids, including TCDCA, GCDCA, TCA, and CDCA showed minor inhibitory effects on the probe substrates of hOAT4 (Fig. 2c and Fig. S1a), which is partly in accordance with the former study (Cha et al., 2000).

Considering the vulnerability and sensitivity of PHTCs against bilirubin, we set the highest concentration as 2.5 $\mu$M in the later 24 hours of culture to attain the bottom cells and the upper medium for estradiol determinations (Fig. 4a-b), the immunostaining, and Western blot conductions (Fig. 5a-b), rather than 10 $\mu$M in the former temporary 3-minute accumulation examinations (Fig. 2-3). We confirmed that bilirubin significantly suppressed the estradiol synthesis and secretion through inhibiting the transporting activity and protein expression of hOAT4 in PHTCs (Fig. 4-5). Previous studies (Samson et al., 2009) have indicated that aromatase, 17 beta-hydroxysteroid dehydrogenase 1 (HSD17$\beta$1), which are highly expressed in placenta are the enzymes responsible for the transformation of DHEAS into E2. The protein and mRNA expressions of those steroidogenic enzymes involved in the synthesis and metabolism (Williams et al., 2002; Niwa et al., 2015; Chatuphonprasert et al., 2018) of estradiol were determined and of little difference (Fig. 5b-c), which suggested that they contributed little to the lowering of estradiol secretion in PHTCs. In addition, it has been reported that OATPs (OATP1A2 and OATP1B1 in the apical membrane and OATP2B1 in the basal membrane) also transport DHEAS (Hagenbuch and Gui, 2008). Although former studies have identified that OATP1A2, OATP1B1, and OATP1B3 contribute to the bilirubin uptake (Briz et al., 2003), they were rarely expressed in the isolated PHTCs (Fig. 5c). The activity of the OATP transporter highly expressed in PHTCs, OATP2B1(Fig. 5c), seemed not to be inhibited by bilirubin (Fig. S2). Additionally, bilirubin dramatically inhibited the DHEAS accumulation in JEG-3 cells in this study (Fig. 3c), which exclusively expressed OAT4 (Fig. S1b). Given the above, we speculated that bilirubin had little influence on the DHEAS uptake by OATPs. Considering that a wide range of endogenous bile acids are substrates or inhibitors of OATPs, bile acids may inhibit both OAT4- and OATP-dependent DHEAS uptake in PHTCs (Fig. 3d).

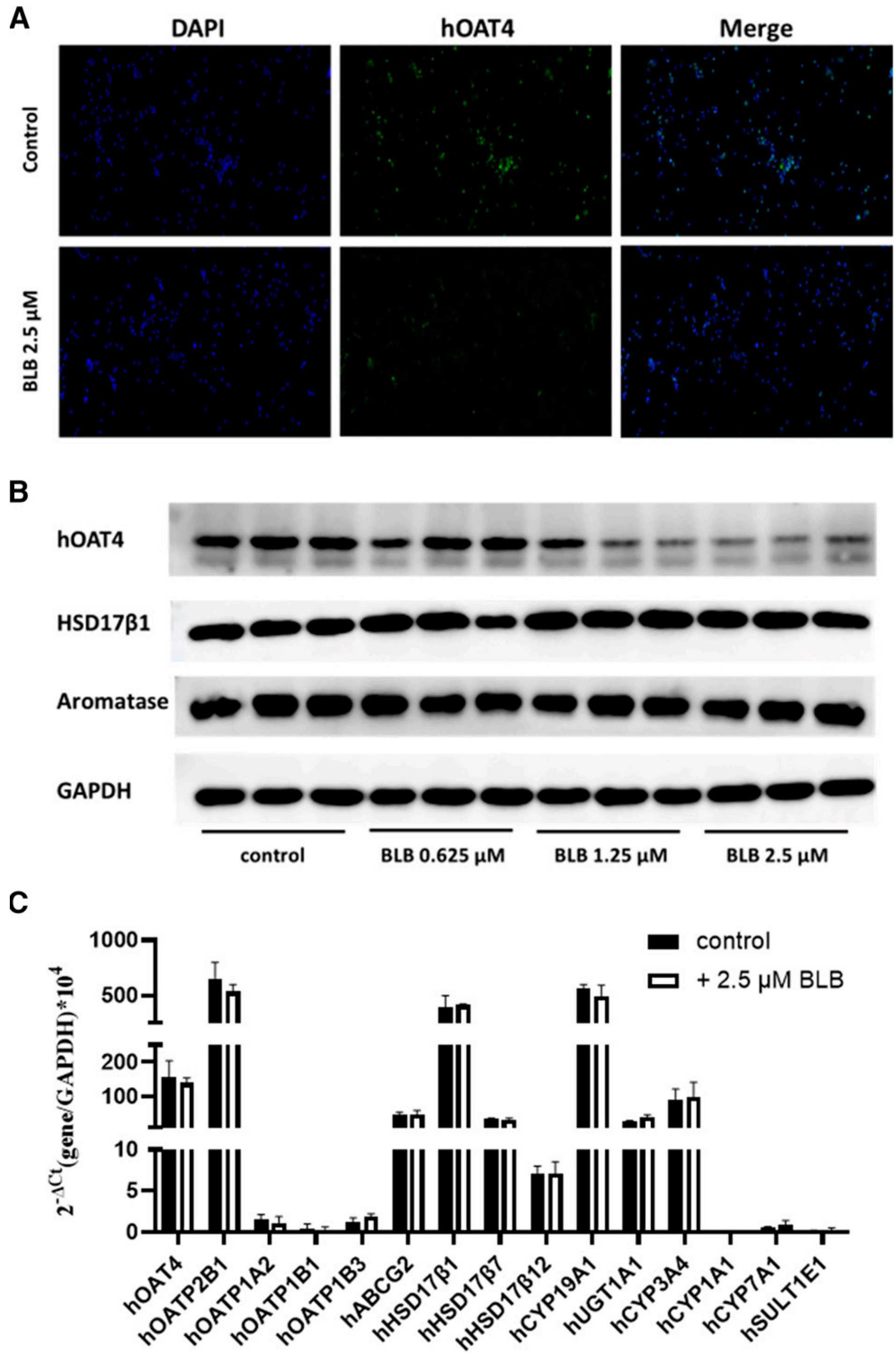


**Fig. 5.** Representative results for (A) immunostaining and (B) Western blot protein expression of human organic anion transporter 4 (hOAT4) in primary human trophoblast cells pretreated with bilirubin. (C) The mRNA expression of human organic anion transporter 4 and the steroidogenic enzymes involved in the synthesis and metabolism of estradiol in primary human trophoblast cells. Relative mRNA levels of target genes were normalized by *human glyceraldehyde-3-phosphate dehydrogenase*, using the ΔCt method and described as $2^{-\Delta Ct}$, ΔCt = average Ct (target gene)-average Ct (glyceraldehyde-3-phosphate dehydrogenase). All cells were pre-incubated with or without bilirubin at 37°C for 24 hours (without dehydroepiandrosterone-3-sulfate) before the performance of immunostaining and Western blot. 4',6-diamidino-2-phenylindole was used to identify nuclei. Data were expressed as mean ± S.D., $n$ = 3. Compared with control cells treated with DMSO. Data were obtained from three independent experiments conducted in triplicate.

Despite the inhibitory roles of those fetal metabolites confirmed above and the overlap of substrates for OATs, they are not substrates of hOAT4 based on their accumulation in mock and hOAT4-CHO cells with or without the existence of the classic OAT inhibitor probenecid. Additionally, bilirubin seems not to have an inhibitory impact on other OAT transporters, including OAT1, OAT2, and OAT3 (Fig. 1i-k), based on the substrate-accumulation study in the absence or presence of bilirubin; thus, bilirubin is potentially a specific inhibitor of OAT4.

Fetal albumin binds to bilirubin, cysteine, free fatty acids, calcium, and drugs, with its concentration serving as a marker of nutritional status. Recently, studies focusing on the measurement of maternal serum ischemia-modified albumin (IMA) and fetal cord-blood IMA concentrations have suggested IMA as simple, novel, and inexpensive marker of oxidative stress status in preeclampsia patients (van Rijn et al., 2008; Rossi et al., 2013; Seshadri Reddy et al., 2018). The disruption of the synthesis of fetal albumin or unintended IMA may lead to a disorder of unconjugated bilirubin concentrations, thus causing hOAT4 activity and expression inhibition.

Maintenance of a healthy pregnancy is dependent on a coordinated sequence of events, including synchrony between the development of the early embryo and establishment of a receptive endometrium. Estradiol and estriol concentrations in maternal serum and urine rise steadily from the first trimester until delivery and diminish rapidly in the postpartum period (Kuijper et al., 2013), and to achieve this, syncytiotrophoblasts require efficient DHEAS and 16α-OH-DHEAS uptake at the basal membrane (BM) facing the fetal circulation. Increased production of estrogen in human placenta during pregnancy is closely associated with parturition; therefore, maternal estradiol and estriol levels are frequently used as quad marker screening to monitor for placental and fetal abnormalities. Preeclampsia with the feature of high blood pressure occurs in 3 to 7% of pregnancies and is one of the main causes of maternal and fetal/neonatal morbidity and mortality. Levels of estrogens, including E1 (Jobe et al., 2013), E2 (Smith et al., 2009; Bussen and Bussen, 2011; Jobe et al., 2013; Yin et al., 2013), and E3 (Smith et al., 2009; Hertig et al., 2010; Jobe et al., 2013), their precursors, such as DHEAS (Hertig et al., 2010), and their byproducts were suppressed in the plasma of women with pregnancy-related hypertensive diseases. Furthermore, E2 and E3 levels were significantly lower (Acikgoz S, et al., 2013) in preeclampsia placental tissues than in tissues from women with normal pregnancies. Although few studies have focused on monitoring the fetal/cord blood bilirubin levels during physiologic pregnancy or pathologic complications, we did observe a trend of higher bilirubin level in the umbilical cord blood from preeclampsia pregnancies (Catarino et al., 2009). The estrogen deficiency occurring during those multisystem disorders (Leslie et al., 2000; Troisi et al., 2003; Wang et al., 2011; Acikgoz S, et al., 2013; Kuijper et al., 2013; Pařízek et al., 2016; Berkane et al., 2017) mentioned above strengthens our assumptions that impaired fetal albumin synthesis or abnormally higher bilirubin concentrations may contribute to the lowering levels of estrogen in the unintended preeclampsia development. Additionally, what roles uric acid (an identified substrate and inhibitor for hOAT4), bilirubin, or bile acid levels in preeclampsia along with maternal hyperuricemia (Lam et al., 2005; Powers et al., 2006; Khaliq et al., 2018; Ryu et al., 2019), hyperbilirubinemia (Duraiswamy et al., 2017) or intrahepatic cholestasis (Raz et al., 2015; Liu et al., 2020) conditions play in the placental hOAT4 activity, expression, estrogen levels, and pregnancy progression remain to be further explored.

Considering the essential role of hOAT4 in the uptake of estriol precursor 16α-OH DHEAS, we have made every effort to get in touch with all kinds of suppliers seeking for possible commercialized 16α-OH-DHEAS, but failed to attain it. The intriguing role of fetal metabolites on hOAT4-mediated uptake of 16α-OH-DHEAS for later estriol synthesis remains to be illuminated.

In conclusion, this study demonstrated that bilirubin reduced the uptake of estrogen precursors and the followed synthesis of estradiol in PHTCs *via* inhibition and downregulation of OAT4. More profound studies concerning specific roles of hOAT4 in the development of preeclampsia or other gestational complications remain to be further illustrated.

## Ethical standards

The primary human trophoblast cell studies have been approved by the Ethics Committee of Affiliated Hangzhou First People's Hospital, College of Pharmaceutical sciences, Zhejiang University. And all pregnant women signed their informed consent prior to the experiment.

## Acknowledgments

We thank Haihong Hu for managing some instruments.

## Authorship Contributions

*Participated in research design:* Zhang, Jiang.

*Conducted experiments:* Zhang, Chen, Dai, Bai, Lu.

*Performed data analysis:* Zhang, Zhou, Jiang.

*Wrote or contributed to the writing of the manuscript:* Zhang, Jiang.

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# Bilirubin Reduces the Uptake of Estrogen Precursors and the Followed Synthesis of Estradiol in Human Placental Syncytiotrophoblasts *via* Inhibition and Down-regulation of OAT4

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**Running title**: Bilirubin inhibits and down-regulates OAT4 in PHTCs

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## Supplemental Materials

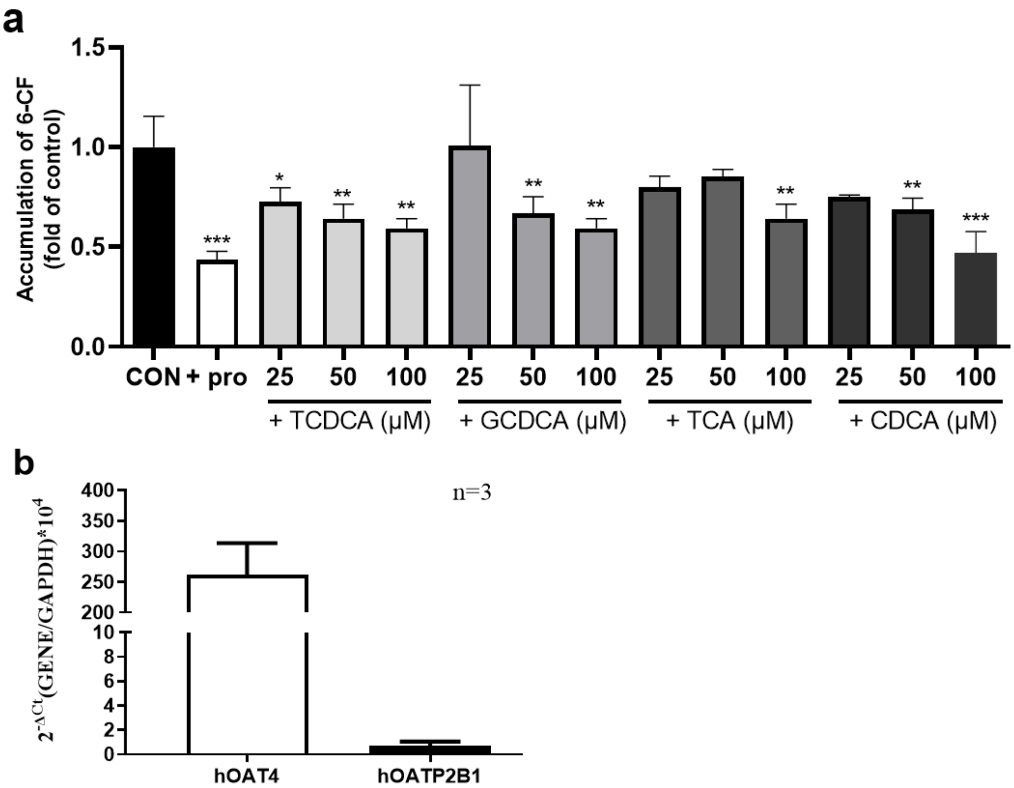


**Fig. S1** Inhibitory effects of bile acids on hOAT4-mediated uptake of 6-CF (5 μM) (a) and mRNA expression of OAT4 and OATP2B1 (b) in JEG-3 cell lines. Bile acids including TCDCA, GCDCA, TCA and CDCA performed relatively weaker inhibitory effects on the accumulation of 6-CF in JEG-3 cells. Compared with the accumulation without inhibitors, *P < 0.05, **P < 0.01, ***P < 0.001. All cells were incubated at 37 °C for 3 minutes. The accumulation was expressed as fold of substrate accumulation without those inhibitors. Data were expressed as mean ± SD from three independent experiments conducted in triplicate.

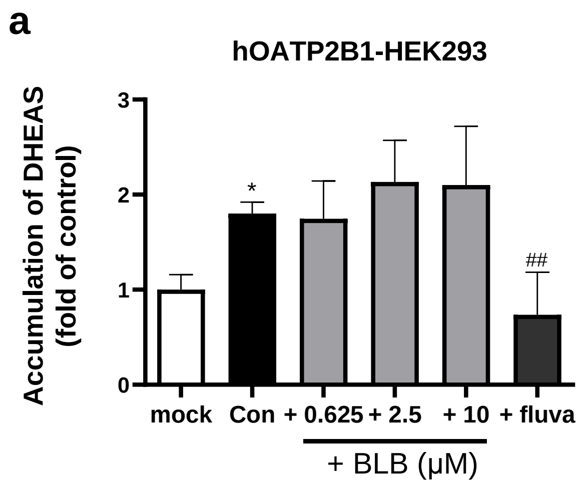


**Fig. S2** Identifications of the bilirubin inhibitory effects on OATP2B1-HEK stably transfected cells constructed and preserved in our laboratory in the presence of 10 μM HSA buffer. Function of tested OATP2B1 cells has been identified by its probe substrate (10 μM DHEAS). Compared with the mock cells, $^{*}P < 0.05$; compared with the hOATP2B1-HEK cells without fluvastatin (a classic inhibitor of OATP2B1), $^{\#\#}P < 0.01$. All cells were incubated at 37 °C for 3 minutes. The accumulation was expressed as fold of substrate accumulation without those inhibitors. Data were expressed as mean ± SD from three independent experiments conducted in triplicate.