Evolutionary analysis of SLC10 family members and insights into function and expression regulation of lamprey NTCP

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Abstract

The Na (+)-taurocholate cotransporting polypeptide (NTCP) is a member of the solute carrier family 10 (SLC10), which consists of 7 members (SLC10a1-SLC10a7). NTCP is a transporter localized to the basolateral membrane of hepatocytes and is primarily responsible for the absorption of bile acids. Although mammalian NTCP has been extensively studied, little is known about the lamprey NTCP (L-NTCP). Here we show that L-NTCP follows the biological evolutionary history of vertebrates, with conserved domain, motif and similar tertiary structure to higher vertebrates. L-NTCP is localized to the cell surface of lamprey primary hepatocytes by immunofluorescence analysis. HepG2 cells overexpressing L-NTCP also showed the distribution of L-NTCP on the cell surface. The expression profile of L-NTCP showed that the expression of NTCP is highest in lamprey liver tissue. L-NTCP also has the ability to transport bile acids, consistent with its higher vertebrate paralogs. Finally, using a farnesoid X receptor (FXR) antagonist, RT-qPCR and flow cytometry results showed that L-NTCP is negatively regulated by the nuclear receptor FXR. This study is important for understanding the adaptive mechanisms of bile acid metabolism after lamprey biliary atresia based on understanding the origin, evolution, expression profile, biological function and expression regulation of L-NTCP.

Introduction

The 10th solute transporter subfamily (SLC10) contains 7 members in mammals, namely Slc10a1, Slc10a2, Slc10a3, Slc10a4, Slc10a5, Slc10a6 and Slc10a7 [1, 2]. Slc10a1, also known as Na (+)-taurocholate cotransporting polypeptide (NTCP), is a 38KDa transporter protein localized to the basal membrane of the liver and is responsible for the uptake of bile acids into hepatocytes [3–6]. Slc10a2, also known as ASBT (apical sodium-bile acid transporter), is highly expressed in the terminal ileum and can efficiently transport both conjugated and unconjugated bile acids[7, 8]. As the functions of Slc10a1 and Slc10a2 are related to bile acids transport, the SLC10 subfamily is also known as the bile acids transport family. Slc10a6, also known as SOAT (sodium-dependent organic anion transporter), does not transport bile acids but rather sulfated sodium taurocholate and steroid metabolites[9, 10]. The functions of Slc10a3, Slc10a4, Slc10a5, and Slc10a7 are not yet known and are referred to as orphan transporter [1, 2].

NTCP, first identified from rat hepatocytes in 1978 [11], was the founding member of the SLC10A gene family[2, 5] and is named Slc10a1 [12]. NTCP transports multiple substrates. In addition to bile acids, it also transports steroids and thyroid hormones, drugs, and drugs conjugated with bile acids [5, 13]. In addition, NTCP is the receptor for the HBV and HDV entry into hepatocytes [14, 15]. Therefore, NTCP has become a drug target for the prevention of viral infection. Although mammalian NTCP has been extensively studied, little is known about lamprey NTCP (L-NTCP).

Lamprey belong to the the Vertebrata, Cyclostomata, is the most primitive vertebrates in existence. They are the living descendants of the world's most ancient jawless vertebrate, which lived 358 million years ago [16]. The life cycle of lamprey is complex and includes larval, metamorphic, juvenile and non-trophic

adult stages [17]. During metamorphosis, the intra- and extrahepatic bile ducts degenerate and are ultimately lost [18–20]. This morphological change is similar to biliary atresia in humans. The biliary system is important for maintaining the metabolic homeostasis of bile acids in the liver tissue. Bile acids are produced from cholesterol in the liver and secreted via the gallbladder into the duodenum, from where they will flow down the intestinal lumen where they are efficiently reabsorbed and circulated back into the portal vein and then transported to hepatocytes in the liver in a process called the enterohepatic circulation of bile acids. The disappearance of the biliary system will impair the efflux of bile acids into the bile duct and cause excessive accumulation of bile acids in the liver, leading to cholestasis and subsequent liver injury [21, 22]. In contrast to human biliary atresia, the lamprey was able to grow and develop normally without liver Cirrhosis or liver failure after biliary atresia [23–25]. This suggests that it has an adaptive mechanism of bile acid metabolism after biliary atresia.

Since NTCP plays an important role in the enterohepatic circulation by transporting bile acids from the portal vein into hepatocytes, the expression level of NTCP determines the level of bile acids in hepatocytes. Therefore, understanding the origin, evolution, biological function, and expression regulation mechanism of L-NTCP in hepatocytes is important for understanding the adaptive mechanism of bile acid metabolism after lamprey biliary atresia.

In this paper, we first revealed the evolutionary relationship of the SLC10 subfamily to which L-NTCP belongs and explored its cellular localization and biological function in lamprey hepatocytes. Finally, we demonstrated for the first time the negative regulation of L-NTCP expression by lamprey farnesoid X receptor (L-FXR). From the above analysis, we suggest that after lamprey biliary atresia, bile acid uptake may be reduced by inhibiting the up-regulated expression of L-NTCP by L-FXR, thereby reducing cholestasis after biliary atresia. This may be an adaptive mechanism of bile acid metabolism after lamprey biliary atresia.

Materials and methods

Experimental animals

Lamprey (*Lampetra japonicum*) in juvenile stages, including males and females come from laboratory artificial fertilization and breed. Every thirty lampreys were maintained in 200-L tanks of recirculating water at 4–6°C. Lampreys handling and all of the experimental procedures were approved by the Animal Welfare and Research Ethics Committee of Liaoning Normal University and Dalian Medical University (Permit Number: SYXK2004-0029).

Identification of lamprey NTCP

We used *Petromyzon marinus* NTCP gene sequences as a query to blast against our lamprey (*Lampetra japonicum*) genome database to obtain L-NTCP sequence information. The full-length open reading frame (ORF) of the L-NTCP gene was obtained by PCR. PCR primers used for the amplification of L-NTCP were designed by Primer Premier 5.0 according to the L-NTCP sequence.

Phylogenetic analysis, conserved motifs and domains analysis

The amino acid sequences of SLC10 subfamily members from different species were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). The phylogenetic tree of SLC10 subfamily was constructed by neighbor-joining method using MEGA X software with the parameters of pairwise-deletion option, p-distance model and 1000 bootstrapped replicates. The accession number used to construct the phylogenetic tree are listed in Supplementary table 1. The analysis of motifs was predicted by MEME (http://meme-suite.org/tools/meme), and the number of motifs was selected as 20. The amino acid sequences of each motif are listed in Supplementary table 2. The domains analysis was predicted by SMART (http://smart.embl-heidelbe rg.de/). In addition, the figure was obtained from ITOL (https://itol.embl.de/).

Synteny construction, gene structure analysis and crystal structure prediction

The synteny analysis was conducted by using the genomicus online tool (http://www.genomicus.biologie.ens.fr), the ensembl database (http://asia.ensembl.org/index.html), and our local lamprey genome databe. Each gene was analyzed in seven species: human, mouse, chicken, lizard, frog, zebrafish and lamprey. The exons and introns of the SLC10 subfamily gene structure analysis were performed using the ensemble database. The protein tertiary structure of human genes was obtained from the PDB database(https://www.rcsb.org). The tertiary structure of lamprey genes were predicted by swiss-model (https://swissmodel.expasy.org/) and superposed with human parologs at superpose website (http://superpose.wishartlab.com/). The tertiary structure of each gene was visualized using VMD software.

Plasmids constructs

The full length coding-sequence (CDS) of L-NTCP was amplified according to the sequence information in our lamprey local genome database. RNA was extracted from lamprey liver tissue for the amplification of L-NTCP. The CDS of L-NTCP was amplified with corresponding primers (Supplementary table 3), cloned into pEASY-T1-simple vector, and sequenced. The CDS of L-NTCP, flanked by *Nhe*I and *Kpn*I sites, was subcloned into pcDNA3.1 for biological functional assay, and flanked by *Hin*dIII and *Bam*HI was subcloned into pEGFP-N1 eukaryotic expression vector for localization assay. L-NTCP fragment, flanked by *Kpn*I and *Hin*dIII, was subcloned into pET-32a prokaryotic expression vector for expression.

Expression of recombinant L-NTCP protein and preparation of its polyclonal antibodies

Recombinant proteins of L-NTCP with His tag were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (Sangon) for 4 h at 37 °C and purified with Ni-NTA His-bind resin column (Sangong). Polyclonal antibodies against recombinant L-NTCP (rL-NTCP) protein (anti-L-NTCP antibodies) were generated by intraperitoneal injection of rL-NTCP protein into adult mice. After five

subsequent injections (100 μg/first time, 50 μg/the next four-time, emulsified with Freund's adjuvant) at 1week intervals, the mice serum was harvested and bound with protein G column for purification. Antibody titers were detected using enzyme linked immunosorbent assay (ELISA).

Immunohistochemistry

Paraffin sections were dewaxed in 100% xylol for 20 min and rehydrated in 100, 90, 80 and 70% graded alcohol solutions. Then, blocking with 3% hydrogen peroxide for 15 min, followed by incubation with normal goat serum working solution for 10 min, the sections were incubated with anti-L-NTCP primary polyclonal antibody (dilution, 1:100) overnight at 4˚C. Mouse IgG (Abcam, dilution, 1:1000) antibody was as control. After washed with PBS, sections were incubated with biotin labeled secondary antibody for 15 min at room temperature, followed by incubation with HRP conjugated streptavidin for 15 min at room temperature. Color development was then performed using a DAB substrate kit (Beyotime Institute of Biotechnology). After counterstaining with hematoxylin, dehydrated and sealed, the sections were observed by light microscopy.

Primary lamprey hepatocytes isolation

After being washed with ice-cold PBS three times, liver tissue was cut into small pieces with sterilized scissors and digested with collagenase (0.15% in DMEM) for 15 min at room temperature. After centrifugation at 3000 rpm for 10 min, cells were collected and cultured in DMEM medium supplemented with 1% penicillin/streptomycin and 2% serum. Transfection of L-NTCP siRNA into lamprey hepatocytes was performed according to the manufacturer’s protocol.

Immunofluorescence

Lamprey Primary hepatocytes were fixed with 4% paraformaldehyde for 15 min and blocked with 5% bovine serum albumin for 1 h. After overnight incubation with L-NTCP antibody, secondary antibodies (Alaxia Fluor 488 goat anti mouse IgG, Abmart) were used for visualization. DAPI (0.5 μg/ml) was used for nuclear counterstaining. Images were obtained using Laser-scanning confocal microscopy (Carl Zeiss LSM710, Germany).

Flow cytometry analysis

Lamprey hepatocytes were fixed with 4% paraformaldehyde for 15 min at room temperature. Cell suspensions were immunostained with L-NTCP antibody and fluoroscein isothiocyanate (FITC)-conjugated anti-rat secondary antibody. Cell samples were then performed by flow cytometry analysis.

HepG2 cell culture and transfection

HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere at 37˚C containing 5% CO_2. Transfection of pDNA3.1-L-NTCP and pEGFP-N1-L-NTCP into HepG2 cells were performed according to the manufacturer’s protocol.

Bile acids treatment in vivo and in vitro

For the in vivo study, TCA was administered by intraperitoneal injection at 20 mg/kg for 24hrs. Injection of phosphate buffered saline (PBS) (50 μL/each) was used as control. For the in vitro study, primary hepatocytes were harvested after treatment with different concentrations of TCA, CA, CDCA and DCA for 24 hrs.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated using TRIzol (Sangon), converted to complementary DNA (cDNA) by HiScript III 1st Strand cDNA Synthesis Kit (Vazyme). RT-qPCR was performed by using ChamQ® Universal SYBR qPCR Master Mix (Vazyme). Primer sequences were described in Supplementary File 2. Amplification was carried out with an initial step at 95°C for 30 sec, followed by 40 cycles of amplification (95°C for 10 sec, 60°C for 30 sec) by using a CFX96 qPCR system (Bio-Rad). GAPDH was used as an internal control, and data were expressed as the ratio of target mRNA to GAPDH mRNA. All results were representative of at least three independent experiments.

Statistical analysis

All data were expressed as mean ± standard error. The student's t-test was performed to determine statistical differences between groups using the GraphPad Prism 7.0. $p < 0.05$ was considered statistically significant.

Results

Phylogenetic trees, conserved motifs and domains analysis

Based on the topology of phylogenetic tree (Fig. 1), the genes of the SLC10 family were divided into three clusters. Slc10a1, Slc10a2, Slc10a4 and Slc10a6 were clustered into one group. Slc10a3 and Slc10a5 clustered in another group. While Slc10a7 belongs to a separate group. Slc10a7 is in the outgroups of the phylogeny tree, indicating Slc10a7 may be the ancestral gene of the family and for the differentiation of other member genes. Styela Clava Slc10a1 and Slc10a2 are present in the outgoups of the vertebrates Slc10a1, Slc10a4 and Slc10a2, Slc10a6 respectively, there is a possibility that the replication of Slc10a1 produce Slc10a4, and replication of Slc10a2 produce Slc10a6. Furthermore, lamprey Slc10a1, Slc10a2, Slc10a3 and Slc10a7 are all located at the bottom of the vertebrate branch. In conclusion, SLC10 family genes follow evolutionary history. SLC10 family member has the only classical SBF domain. By analyzing the composition of domain motif, we found that motif 5, 6, 9, and 20 form the SBF domain in the Slc10a7 subgroup, while motifs 1, 3, 4, and 7 form the SBF domain in the other two subgroups. Each subgroup members have a similar motif composition. However, in the subgroup consisting of Slc10a1 and Slc10a4, Slc10a4 contains motif13, whereas Slc10a1 does not. In the subgroup consisting of Slc10a2 and Slc10a6, Slc10a2 contains motif15 but Slc10a6 does not. The motif composition of L-Slc10a1 and L-Slc10a7 is consistent with other higher vertebrates and is highly conserved. In contrast,

the motif composition of L-Slc10a2 and L-Slc10a3 is not completely consistent, as shown by the fact that L-Slc10a2 does not contain motif14, whereas L-Slc10a3 does.

Synteny and gene structure analysis of Lamprey SLC10 genes

Seven different species, including human, mouse, chicken, lizard, frog, zebrafish, and lamprey, were used to analyze the genetic environment of Lamprey *SLC10* genes. As shown in Fig. 2, *SLC10* genes form conserved linkage group with the surrounding genes in the genome of higher vertebrates, but not in lamprey. However, we found that consistent with the *Slc10a1* and *Slc10a7* gene in higher vertebrates respectively, *L-Slc10a1* has a colinear relationship with *COX16*.gene, L-Slc10a7 has a colinear relationship with *TTC29* gene. Next, different species such as human, mouse, chicken, lizard, frog, zebrafish, and lamprey were used to analyze the gene structure of Slc10 genes. As shown in Fig. 3. the structure of Slc10 genes is relatively stable. The number of exons of Slc10 gene in lamprey was almost similar to that of other higher vertebrates.

Tertiary structure prediction of Slc10 genes

By comparing the tertiary structures of Slc10a1 and Slc10a7 in lamprey with those in humans, we found that no significant differences were observed when the tertiary structure of L-Slc10a1 was superposed with H-Slc10a1 (Fig. 4), L-Slc10a7 superposed with H-Slc10a7 (Fig. 4), with root mean square deviation (RMSD) as $3A^0$ and $2.17A^0$ respectively, indicating that L-Slc10a1 and L-Slc10a7 have similar structures to their orthologs and may have a similar function as in higher vertebrates.

Tissue distribution and subcellular locations of L-NTCP

The expression profile of L-Slc10a1(L-NTCP) was determined by RT-qPCR. As shown in Fig. 5A, the expression level of L-NTCP is the highest in the liver, followed by kidney, gonads, and intestine. The expression level of L-NTCP is the lowest in the heart, gills, and supraneural body. Next, immunohistochemistry was performed to examine the tissues distribution of L-NTCP. As shown in Fig. 5B, compared to the isotype control, L-NTCP was specifically expressed in the liver, kidney, intestine, and brain. The results of immunofluorescence using anti-L-NTCP antibodies showed that L-NTCP was located on the cell membrane of lamprey hepatocytes (Fig. 5C). Next, transfection of recombinant eGFP-tagged NTCP vector (pEGFP-N1-L-NTCP) into HepG2 cells also revealed its predominant localization at the plasma membrane (Fig. 5D).

L-NTCP promotes the absorption of bile acids in lamprey hepatocytes cell

Bile acids are endogenous ligands of FXR. Thus bile acids absorbed by NTCP into the cells are able to activate FXR and thus increase the expression level of FXR. Therefore, we can detect the effect of L-NTCP on bile acid uptake by detecting changes in the expression of L-FXR. RT-qPCR results showed that compared to control, L-FXR expression was increased in the presence of 50–250 μM CA, 10–50 μM

CDCA, 25μM DCA and 50 μM TCA (Fig. 6A). A peak expression level for L-FXR was observed when the cell was treated with 250 μM CA, 50 μM CDCA, 25μM DCA and 50 μM TCA for 24 hrs. Next, siRNA-L-NTCP was used to knock down the expression level of L-NTCP. RT-qPCR and flow cytometry results showed that siRNA-L-NTCP caused an 50% and 30% reduction in L-NTCP mRNA and protein levels respectively (Fig. 6B,C). Under L-NTCP knockdown, 50 μM TCA and 250 μM induced up-regulation of L-FXR decreased significantly, compared to scrambled siRNA (Fig. 6D). Similarly, in vivo experiments showed that the expression of L-FXR and its target genes was significantly upregulated after 24 hrs treatment with TCA by intraperitoneal injection (Fig. 6E). The above experiments demonstrated that L-NTCP has a biological role in the uptake of bile acids, which is consistent with higher vertebrates.

L-NTCP promotes the absorption of bile acids in HepG2 cell

We transfected a eukaryotic expression vector of pcDNA3.1-L-NTCP for the analysis of L-NTCP function. Transient overexpression of NTCP resulted in an increase in the expression of FXR and its target genes SHP, BSEP, and MDR3 with the treatment of TCA in a concentration dependant manner (Fig. 6F), indicating that L-NTCP has the function of absorbing bile acids.

Regulation of L-NTCP expression by L-FXR

Our previous experiments demonstrated that L-NTCP expression was significantly down-regulated, whereas L-FXR expression was significantly up-regulated after biliary atresia in lamprey, indicating that L-NTCP may be negatively regulated by L-FXR, which is consistent with higher vertebrates. To determine whether L-FXR has a negative regulatory effect on L-NTCP in lamprey, the FXR antagonist guggulsterone was used to stimulate lamprey hepatocytes for 2hrs before the treatment of 50 μM TCA for 24hrs. As shown in Fig. 7A, the pretreatment with guggulsterone reduced the expression of L-FXR, while increased the expression of L-NTCP, induced by 50 μM TCA (Fig. 7A). Flow cytometry also showed the pretreatment with guggulsterone increased the expression of L-NTCP (Fig. 7B), indicating that L-FXR plays a negative regulatory role in L-NTCP expression in lamprey.

Discussion

Lamprey is the most primitive jawless vertebrates. The biological role of lamprey NTCP has not been reported, although it has been extensively studied in higher vertebrates. In order to understand the biological functions of L-NTCP, phylogenetic tree of SLC10 family members was first constructed, and their domain and motif were predicted and used for comparative analysis. As seen in Fig. 1, Slc10a7 is localized at the outgoups of the phylogeny tree. In addition, the motif composition of Slc10a7 is more different from other members, indicating that Slc10a7 may be the ancestral genes of the family and for the differentiation of other member genes. Although Slc10a1, Slc10a2, Slc10a4, Slc10a6 was emerged from Slc10a7, but their transport activities and transported substrates are different [26]. We speculate that their transport function may have been altered in the process of ancestral gene differentiation. L-NTCP localize the bottom of the SLC10A1 clade, demonstrating that it is the primitive form for vertebrates.

What's more, the motif composition of L-NTCP is the same as that of other orthologs in higher verterbrates. The tertiary structure of L-NTCP is similar to that of H-NTCP, with an RMSD value of 3. Based on the reported conserved amino acid loci of NTCP involved in the transport of bile acids [27], multiple sequence alignments showed that L-NTCP also has the conserved sites for transporting bile acids (Supplementary Fig. 1). Expression profile analysis by RT-qPCR showed that L-NTCP was most abundantly expressed in liver tissue. Next, immunofluorescence using self-made anti-L-NTCP antibodies also showed that L-NTCP localized the surface of lamprey hepatocytes. Overexpression of L-NTCP, expressed by fusion with GFP, in HepG2 cells displayed a green fluorescent signal at the cell surface. The results of bioinformatic analysis, expression profile and cell localization hinted us that L-NTCP may play the same role in bile acids absorption as in humans. However, it is not clear whether L-NTCP has the same function as higher vertebrates in bile acid absorption.

To confirm the function of L-NTCP in bile acid uptake, we first performed RT-qPCR to detect changes in L-FXR expression levels under different types of bile acid treatment. Since bile acids are the endogenous ligands of FXR, the upregulation of FXR expression levels represents bile acid uptake mediated by NTCP [28]. As shown in Fig. 6A, a certain concentration range of bile acids leads to a significant upregulation of L-FXR expression. In contrast, when L-NTCP was knocked down by siRNA, bile acids-induced L-FXR upregulation was significantly decreased. Since lamprey can not recognize the promoter in eukaryotic expression vectors, exogenous genes can not be overexpressed by transfection of eukaryotic expression vectors into lamprey hepatocytes. Overexpression of L-NTCP in HepG2 by transfection of pCDN3.1-L-NTCY was then performed. Compared to control, ectopic expression of L-NTCP resulted in an increase in the expression of FXR and its target genes expression. Finally, intraperitoneal injection of TCA also lead to the upregulation of FXR and its target genes. These findings provide support for the notion that L-NTCP has the ability of taking up bile acids. However, there is a study reporting that the Slc10a1 gene in the marine skate (*Leucoraja erinacea*, skSlca1) is primarily expressed in the brain and testes but not in the liver[29]. Furthermore, it is unlikely that skSlc10a1 is a physiological bile salt transporter, but rather a neurosteroid conjugate transporter. The authors speculated that the substrate specificity of skSlc10a1 for bile salts and the tissue specificity for liver occurred later in vertebrate evolution[29]. However, according to our results, since L-NTCP is highly expressed in liver tissues and also has a role in bile acids uptake, we speculated that the NTCP in lamprey, which represent the most primitive vertebrates, is likely to be the physiological bile salt transporter. It is possible that during the evolution of NTCP in vertebrates, skSlc10a1 was localized in the brain due to physiological needs and its ability to absorb bile acids was gradually reduced, while its role in transporting steroids was maintained. However, the study of Slc10a1 in other species would shed light on these questions.

Our previous study showed that after lamprey biliary atresia, L-NTCP expression was significantly down-regulated, while L-FXR was significantly up-regulated, with a negative correlation between them. It should be noted that this negative regulation of NTCP by FXR has been demonstrated in mouse and human [6, 30]. Bile acids activate the FXR response element in the small heterodimer partner (SHP) promoter leading to the expression of SHP, which in turn interferes with retinoid X receptor (RXR):retinoic acid receptor

(RAR) transactivation of the NTCP promoter, resulting in decreased expression of NTCP [31, 32]. SHP also inhibits HNF1α and HNF4α mediated transcription of NTCP expression [33–35]. Thus, FXR inhibits NTCP mRNA expression through SHP partners. However it is not confirmed whether L-FXR negatively regulate L-NTCP in lamprey. To investigate the expression regulation of L-NTCP by L-FXR, the FXR antagonist guggulsterone was used to treat lamprey hepatocytes. As shown in Fig. 7, guggulsterone increased L-NTCP expression while causing a significant decrease in bile acid-induced L-FXR upregulation expression in a concentration dependent manner. Flow cytometry also showed the similar results, indicating that L-FXR negatively regulates L-NTCP expression, which is consistent with human.

The most striking feature of biliary atresia in humans is that bile secretion and flow are impaired, resulting in a buildup of potentially toxic bile acids in the liver and systemic circulation [24, 36]. Under physiological conditions, bile acids play an important role in lipid digestion and absorption and act as signalling molecules to regulate body metabolism [37, 38]. However, the excessive accumulation of bile acids in the liver tissue will causes oxidative stress and mitochondrial damage in the liver, leading to the release of pro-inflammatory cytokines, that trigger an inflammatory response. Liver parenchymal cells are then infected by recruited neutrophils and other immune cells, resulting in liver fibrosis, cirrhosis, and ultimately liver failure [21]. In contrast to human biliary atresia, lamprey was able to grow and develop normally without severe liver injury after biliary atresia [23–25]. This indicates that lamprey have some adaptive mechanism of bile acid metabolism after biliary atresia. Yeh et al. (2012) and Cai et al. (2013) reported that lampreys adapted to biliary atresia by limiting bile acid synthesis by decreasing the expression of bile acid rate-limiting enzymes in liver tissue, altering the composition of bile salts to reduce bile acid toxicity, increasing bile acid efflux through the kidney, and shifting the site of bile acid synthesis from the liver to the intestine [39, 40]. Since we have demonstrated that the function of L-NTCP is to absorb bile acids, we hypothesized that inhibition of L-NTCP by L-FXR may be another adaptive mechanism after lamprey biliary atresia that is important for reducing hepatic bile acid levels and preventing cytotoxic effects of excess bile acids on hepatocytes.

Declarations

Ethical Approval

The animal experiments were performed in accordance with the regulations of the Animal Welfare and Research Ethics Committee of the Institute of Dalian Medical University's Animal Care protocol (Permit Number: SCXK2008-0002).

Consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no conflict of interest.

Authors' contributions

Yan Chi contributed to the study conception and design. Yingying Zhu prepared Fig.1-5. Qipeng Zhang prepared Fig.6-7. Primary cell isolation was performed by Jilong Pan and Jindi Liu. Molecular docking was performed Lei Qian. Material preparation were performed by Tiesong Li and Hao Wang. Ting Zhu helped us to analyze the phylogenetic tres. Yue pang and Qingwei Li provided the lab condition. The manuscript was written by Yan Chi and Qipeng Zhang. Yan chi will revise the manuscript. All of the authors reviewed the manuscript and approved the final manuscript.

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Availability of data and materials

All authors make sure that all data and materials support published claims and comply with field standards.

References

1. Anwer MS, Stieger B: Sodium-dependent bile salt transporters of the SLC10A transporter family: more than solute transporters. Pflugers Arch 2014, 466:77–89.
2. Claro da Silva T, Polli JE, Swaan PW: The solute carrier family 10 (SLC10): beyond bile acid transport. Mol Aspects Med 2013, 34:252–269.
3. Ananthanarayanan M, Ng OC, Boyer JL, Suchy FJ: Characterization of cloned rat liver Na(+)-bile acid cotransporter using peptide and fusion protein antibodies. Am J Physiol 1994, 267:G637-643.
4. Stieger B, Hagenbuch B, Landmann L, Höchli M, Schroeder A, Meier PJ: In situ localization of the hepatocytic Na+/Taurocholate cotransporting polypeptide in rat liver. Gastroenterology 1994, 107:1781–1787.
5. Döring B, Lütteke T, Geyer J, Petzinger E: The SLC10 carrier family: transport functions and molecular structure. Curr Top Membr 2012, 70:105–168.
6. Appelman MD, Wettengel JM, Protzer U, Oude Elferink RPJ, van de Graaf SFJ: Molecular regulation of the hepatic bile acid uptake transporter and HBV entry receptor NTCP. Biochim Biophys Acta Mol Cell Biol Lipids 2021, 1866:158960.

7. Shneider BL, Dawson PA, Christie DM, Hardikar W, Wong MH, Suchy FJ: Cloning and molecular characterization of the ontogeny of a rat ileal sodium-dependent bile acid transporter. J Clin Invest 1995, 95:745–754.
8. Craddock AL, Love MW, Daniel RW, Kirby LC, Walters HC, Wong MH, Dawson PA: Expression and transport properties of the human ileal and renal sodium-dependent bile acid transporter. Am J Physiol 1998, 274:G157-169.
9. Geyer J, Döring B, Meerkamp K, Ugele B, Bakhiya N, Fernandes CF, Godoy JR, Glatt H, Petzinger E: Cloning and functional characterization of human sodium-dependent organic anion transporter (SLC10A6). J Biol Chem 2007, 282:19728–19741.
10. Geyer J, Bakhaus K, Bernhardt R, Blaschka C, Dezhkam Y, Fietz D, Grosser G, Hartmann K, Hartmann MF, Neunzig J, et al: The role of sulfated steroid hormones in reproductive processes. J Steroid Biochem Mol Biol 2017, 172:207–221.
11. Anwer MS, Hegner D: Effect of Na on bile acid uptake by isolated rat hepatocytes. Evidence for a heterogeneous system. Hoppe Seylers Z Physiol Chem 1978, 359:181–192.
12. Hagenbuch B, Dawson P: The sodium bile salt cotransport family SLC10. Pflugers Arch 2004, 447:566–570.
13. Faber KN, Müller M, Jansen PL: Drug transport proteins in the liver. Adv Drug Deliv Rev 2003, 55:107–124.
14. Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, et al: Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 2012, 1:e00049.
15. Drexler JF, Geipel A, König A, Corman VM, van Riel D, Leijten LM, Bremer CM, Rasche A, Cottontail VM, Maganga GD, et al: Bats carry pathogenic hepadnaviruses antigenically related to hepatitis B virus and capable of infecting human hepatocytes. Proc Natl Acad Sci U S A 2013, 110:16151–16156.
16. Janvier P: Palaeontology: modern look for ancient lamprey. Nature 2006, 443:921–924.
17. Mathai PP, Byappanahalli MN, Johnson NS, Sadowsky MJ: Gut Microbiota Associated With Different Sea Lamprey (Petromyzon marinus) Life Stages. Front Microbiol 2021, 12:706683.
18. Youson JH, Sidon EW: Lamprey biliary atresia: first model system for the human condition? *Experientia* 1978, 34:1084–1086.
19. Sidon EW, Youson JH: Morphological changes in the liver of the sea lamprey, Petromyzon marinus L., during metamorphosis. II. Canalicular degeneration and transformation of the hepatocytes. J Morphol 1983, 178:225–246.
20. Youson JH, Sidon EW, Peek WD, Shivers RR: Ultrastructure of the hepatocytes in a vertebrate liver without bile ducts. J Anat 1985, 140 (Pt 1):143–158.
21. Cai SY, Boyer JL: The role of bile acids in cholestatic liver injury. Ann Transl Med 2021, 9:737.
22. Copple BL, Jaeschke H, Klaassen CD: Oxidative stress and the pathogenesis of cholestasis. Semin Liver Dis 2010, 30:195–204.

23. Ortiz-Perez A, Donnelly B, Temple H, Tiao G, Bansal R, Mohanty SK: Innate Immunity and Pathogenesis of Biliary Atresia. Front Immunol 2020, 11:329.
24. Vij M, Rela M: Biliary atresia: pathology, etiology and pathogenesis. Future Sci OA 2020, 6:Fso466.
25. Chung-Davidson YW, Yeh CY, Li W: The Sea Lamprey as an Etiological Model for Biliary Atresia. *Biomed Res Int* 2015, 2015:832943.
26. Grosser G, Müller SF, Kirstgen M, Döring B, Geyer J: Substrate Specificities and Inhibition Pattern of the Solute Carrier Family 10 Members NTCP, ASBT and SOAT. Front Mol Biosci 2021, 8:689757.
27. Zakrzewicz D, Geyer J: Multitasking Na(+)/Taurocholate Cotransporting Polypeptide (NTCP) as a Drug Target for HBV Infection: From Protein Engineering to Drug Discovery. Biomedicines 2022, 10.
28. Jones H, Alpini G, Francis H: Bile acid signaling and biliary functions. Acta Pharm Sin B 2015, 5:123–128.
29. Yu D, Zhang H, Lionarons DA, Boyer JL, Cai SY: Na(+)-taurocholate cotransporting polypeptide (NTCP/SLC10A1) ortholog in the marine skate Leucoraja erinacea is not a physiological bile salt transporter. Am J Physiol Regul Integr Comp Physiol 2017, 312:R477-r484.
30. Claudel T, Zollner G, Wagner M, Trauner M: Role of nuclear receptors for bile acid metabolism, bile secretion, cholestasis, and gallstone disease. Biochim Biophys Acta 2011, 1812:867–878.
31. Denson LA, Sturm E, Echevarria W, Zimmerman TL, Makishima M, Mangelsdorf DJ, Karpen SJ: The orphan nuclear receptor, shp, mediates bile acid-induced inhibition of the rat bile acid transporter, ntcp. Gastroenterology 2001, 121:140–147.
32. Zollner G, Wagner M, Fickert P, Geier A, Fuchsbichler A, Silbert D, Gumhold J, Zatloukal K, Kaser A, Tilg H, et al: Role of nuclear receptors and hepatocyte-enriched transcription factors for Ntcp repression in biliary obstruction in mouse liver. Am J Physiol Gastrointest Liver Physiol 2005, 289:G798-805.
33. Geier A, Martin IV, Dietrich CG, Balasubramaniyan N, Strauch S, Suchy FJ, Gartung C, Trautwein C, Ananthanarayanan M: Hepatocyte nuclear factor-4alpha is a central transactivator of the mouse Ntcp gene. Am J Physiol Gastrointest Liver Physiol 2008, 295:G226-233.
34. Jung D, Kullak-Ublick GA: Hepatocyte nuclear factor 1 alpha: a key mediator of the effect of bile acids on gene expression. Hepatology 2003, 37:622–631.
35. Wang B, Cai SR, Gao C, Sladek FM, Ponder KP: Lipopolysaccharide results in a marked decrease in hepatocyte nuclear factor 4 alpha in rat liver. Hepatology 2001, 34:979–989.
36. Hartley JL, Davenport M, Kelly DA: Biliary atresia. Lancet 2009, 374:1704–1713.
37. Di Ciaula A, Garruti G, Lunardi Baccetto R, Molina-Molina E, Bonfrate L, Wang DQ, Portincasa P: Bile Acid Physiology. Ann Hepatol 2017, 16:s4-s14.
38. Perino A, Demagny H, Velazquez-Villegas L, Schoonjans K: Molecular Physiology of Bile Acid Signaling in Health, Disease, and Aging. Physiol Rev 2021, 101:683–731.
39. Yeh CY, Chung-Davidson YW, Wang H, Li K, Li W: Intestinal synthesis and secretion of bile salts as an adaptation to developmental biliary atresia in the sea lamprey. Proc Natl Acad Sci U S A 2012, 109:11419–11424.

40. Cai SY, Lionarons DA, Hagey L, Soroka CJ, Mennone A, Boyer JL: Adult sea lamprey tolerates biliary atresia by altering bile salt composition and renal excretion. Hepatology 2013, 57:2418–2426.

Figures

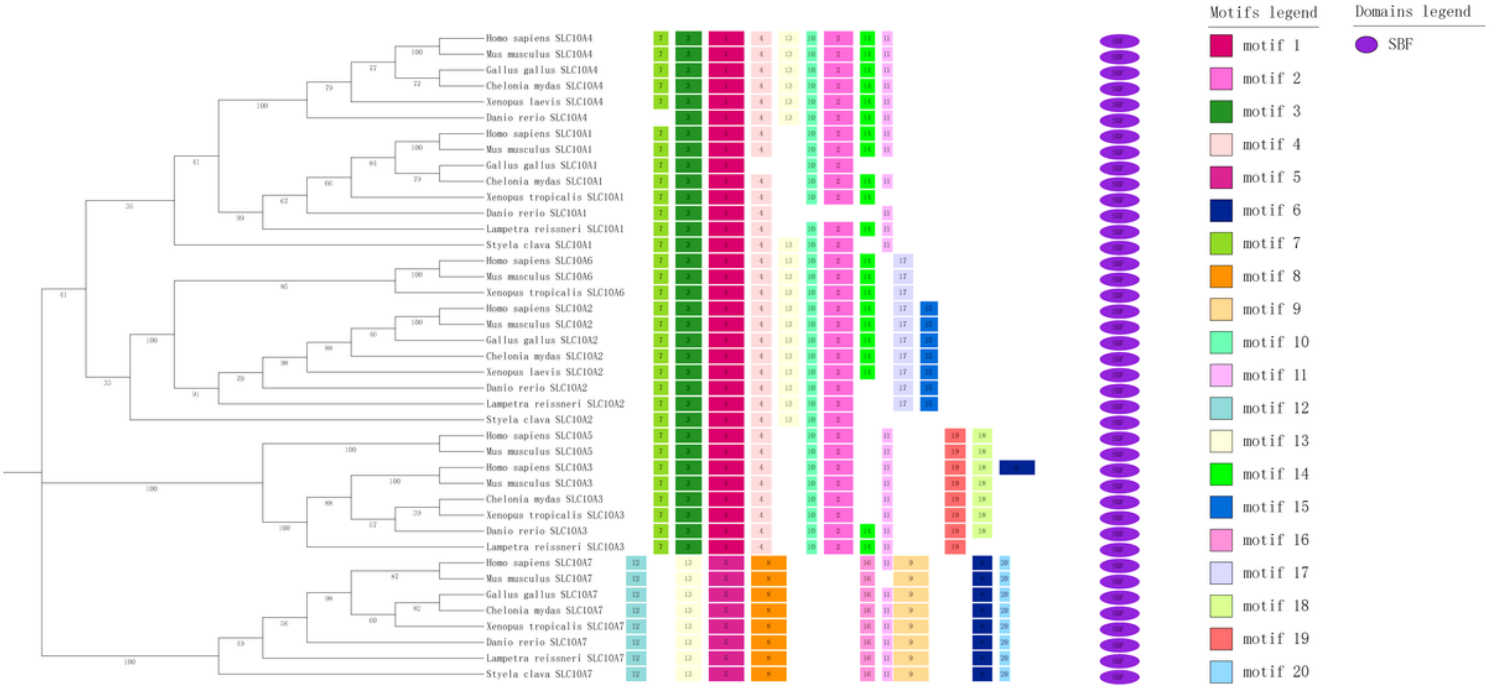

Figure 1

Phylogenetic relationships, conserved protein motifs, and conserved protein domains in SLC10 subfamily.

The phylogenetic tree of the SLC10 subfamily was constructed by neighbour-joining method using MEGA X software with the parameters of pairwise-deletion option, p-distance model and 1000 bootstrapped replicates. The accession number used to construct the phylogenetic tree are listed in Supplementary table 1. Motifs 1-20 are indicated by differently colored boxes. The amino acid sequences of each motif are listed in Supplementary table 2. The motif and protein domain names are labeled on the laterallegend.

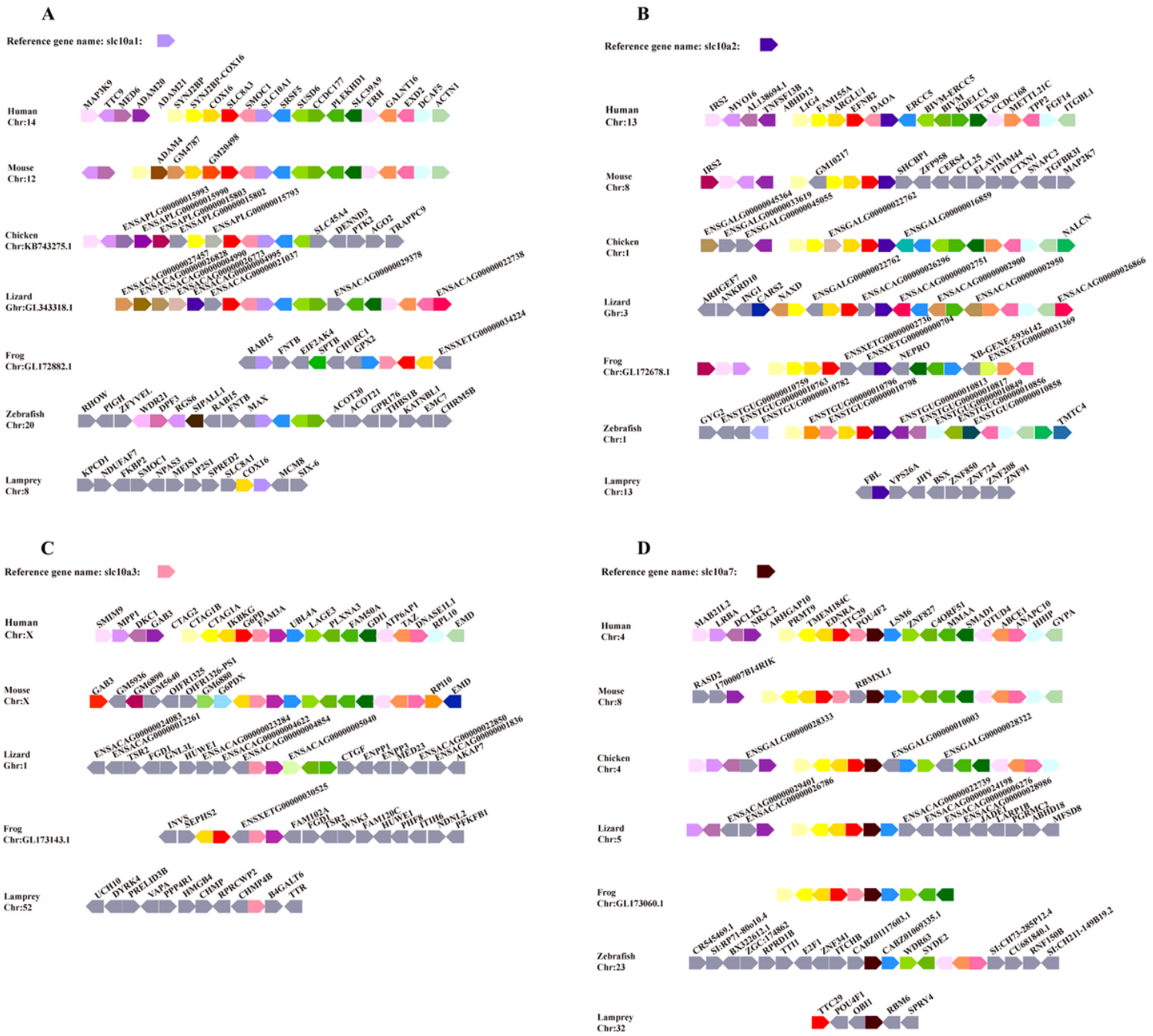

Figure 2

Synteny analysis of SLC10 subfamily genes in different species. A. The neighbourhoods of SLC10a1. B. The neighbourhoods of SLC10a2. C. The neighbourhoods of SLC10a3. D. The neighbourhoods of SLC10a7. Arrows pointing in opposite directions represent a given gene on opposite strands. Square arrows indicate gene position and orientation in the genome; homologous genes shared by species are shown in the same column. Gene neighbourhood and protein sequence data were obtained from the Genomicus and Ensembl databases.

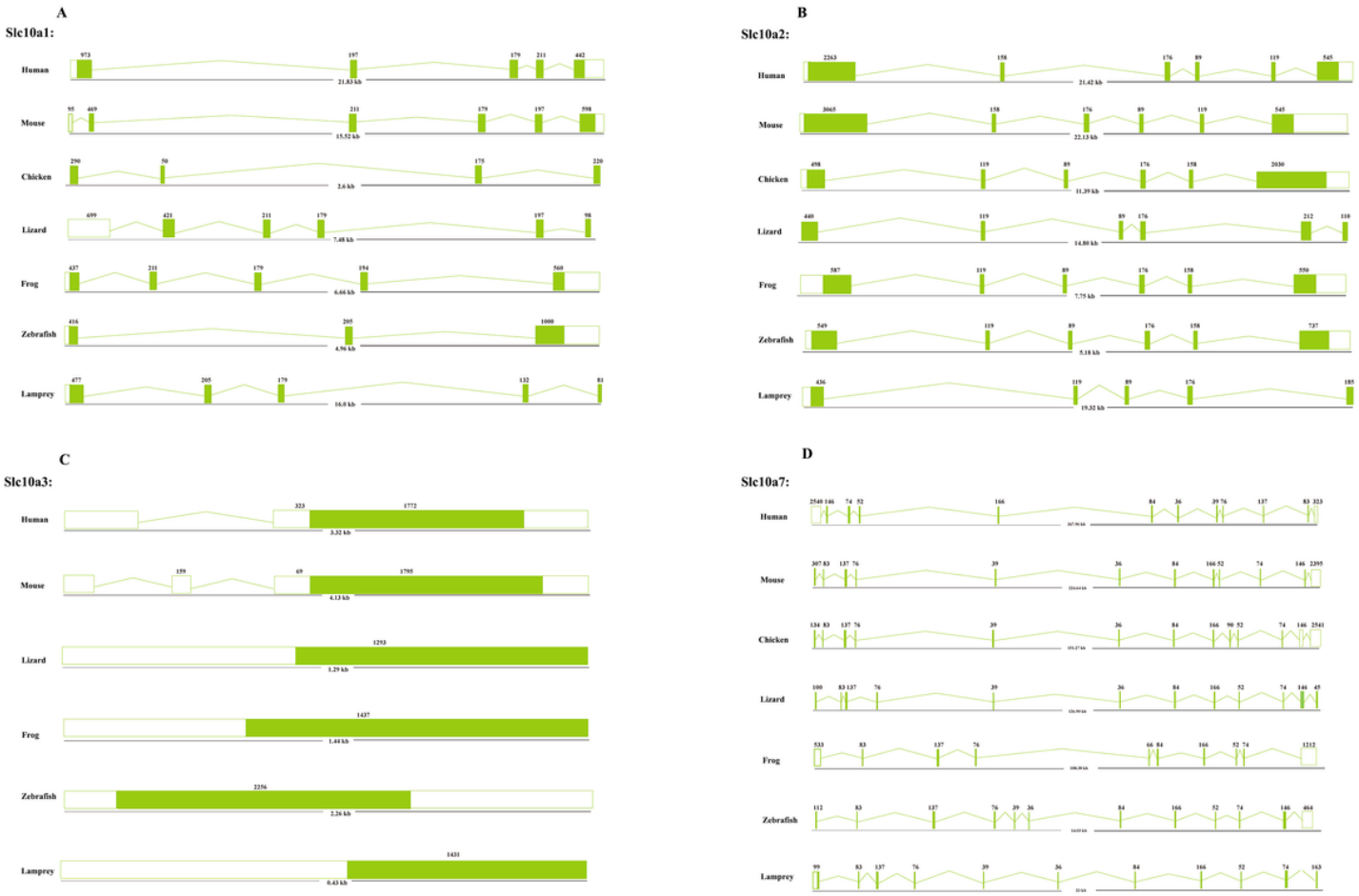

Figure 3

Gene structure analysis of the SLC10 subfamily from representative species. A. SLC10a1 gene structure. B. SLC10a2 gene structure. C. SLC10a3 gene structure. D. SLC10a7 gene structure. The SLC10 subfamily gene sequence in lamprey is non-redundant. Green lines represent introns. Green boxes represent exons. The number indicates exon length. White boxes represent non-coding regions. SLC10 subfamily gene structure data were obtained from the Ensembl database.

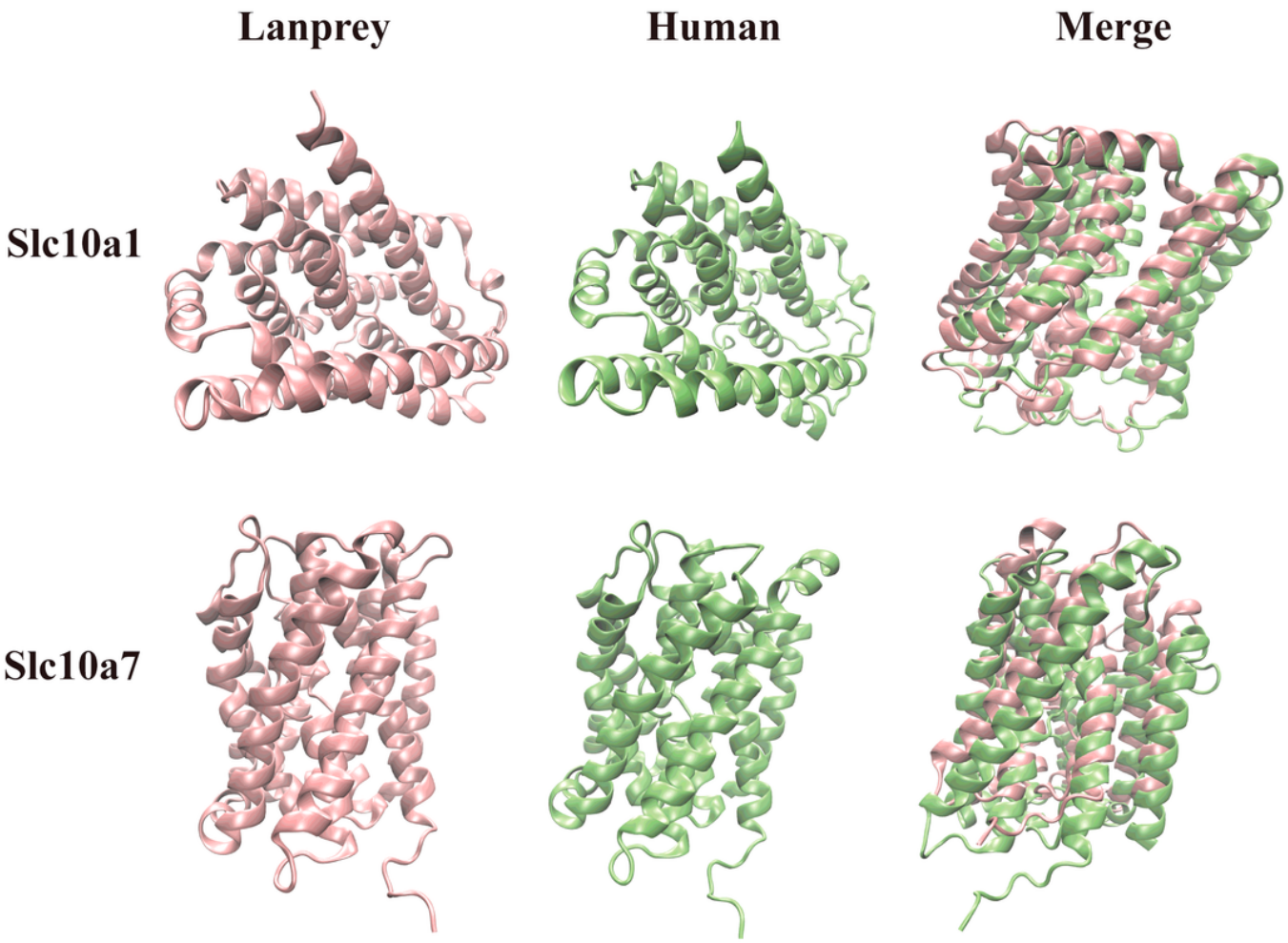

Figure 4

Tertiary structure prediction and comparative analysis of SLC10a1 and SLC10a7. The tertiary structure of lamprey genes were predicted by SWISS-MODEL (https://swissmodel.expasy.org/) and superposed with human parologs at superpose website (http://superpose.wishartlab.com/). Tertiary structure of each genes were visualized by VMD software.

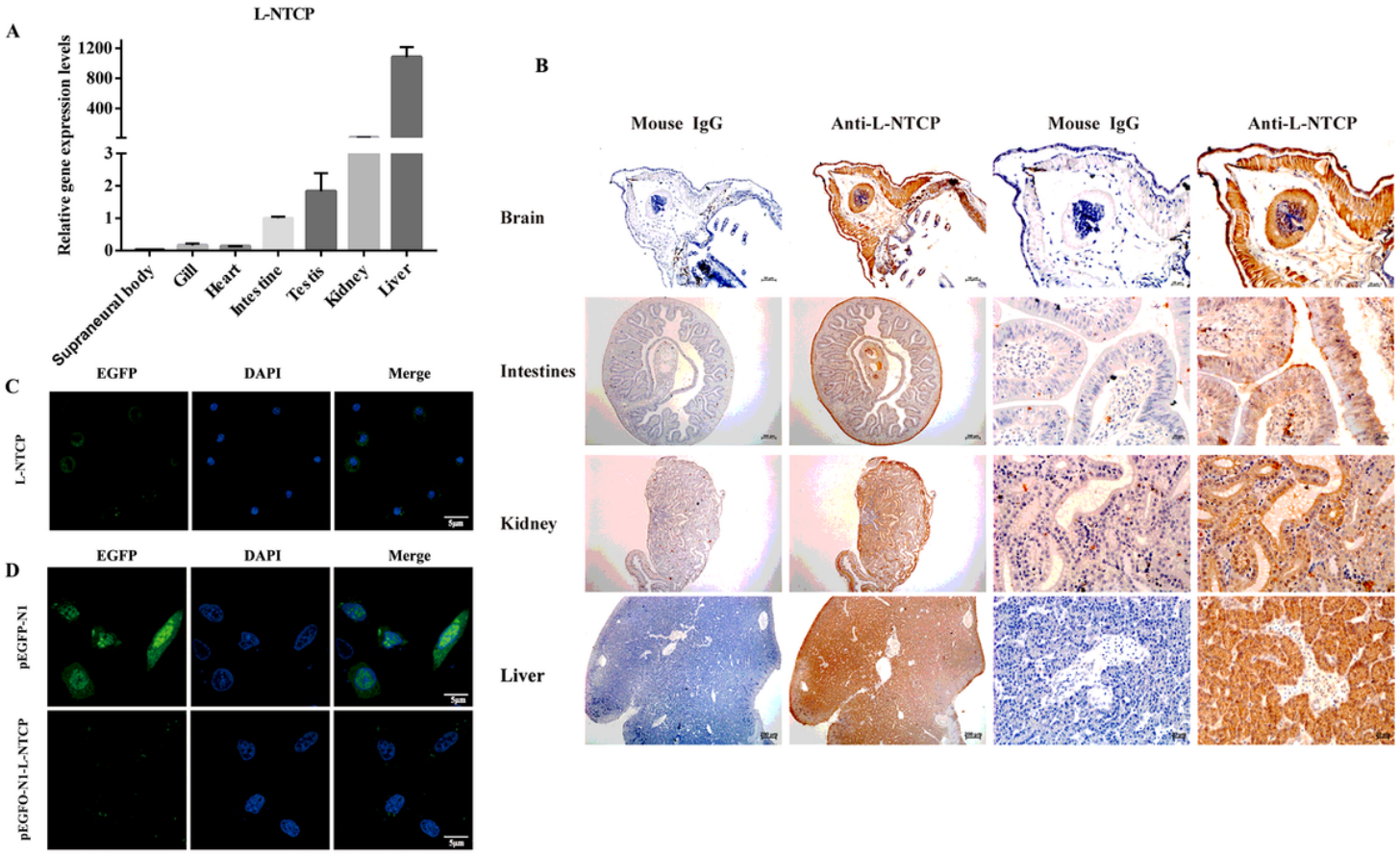

Figure 5

Expression profile and cell localization of L-NTCP. A.Tissue distribution of L-NTCP by RT-qPCR analysis. B. Tissue distribution of L-NTCP by Immunohistochemistry. C. Cell localization of L-NTCP in lamprey hepatocytes. D. Cell localization of L-NTCP in HepG2 cells.

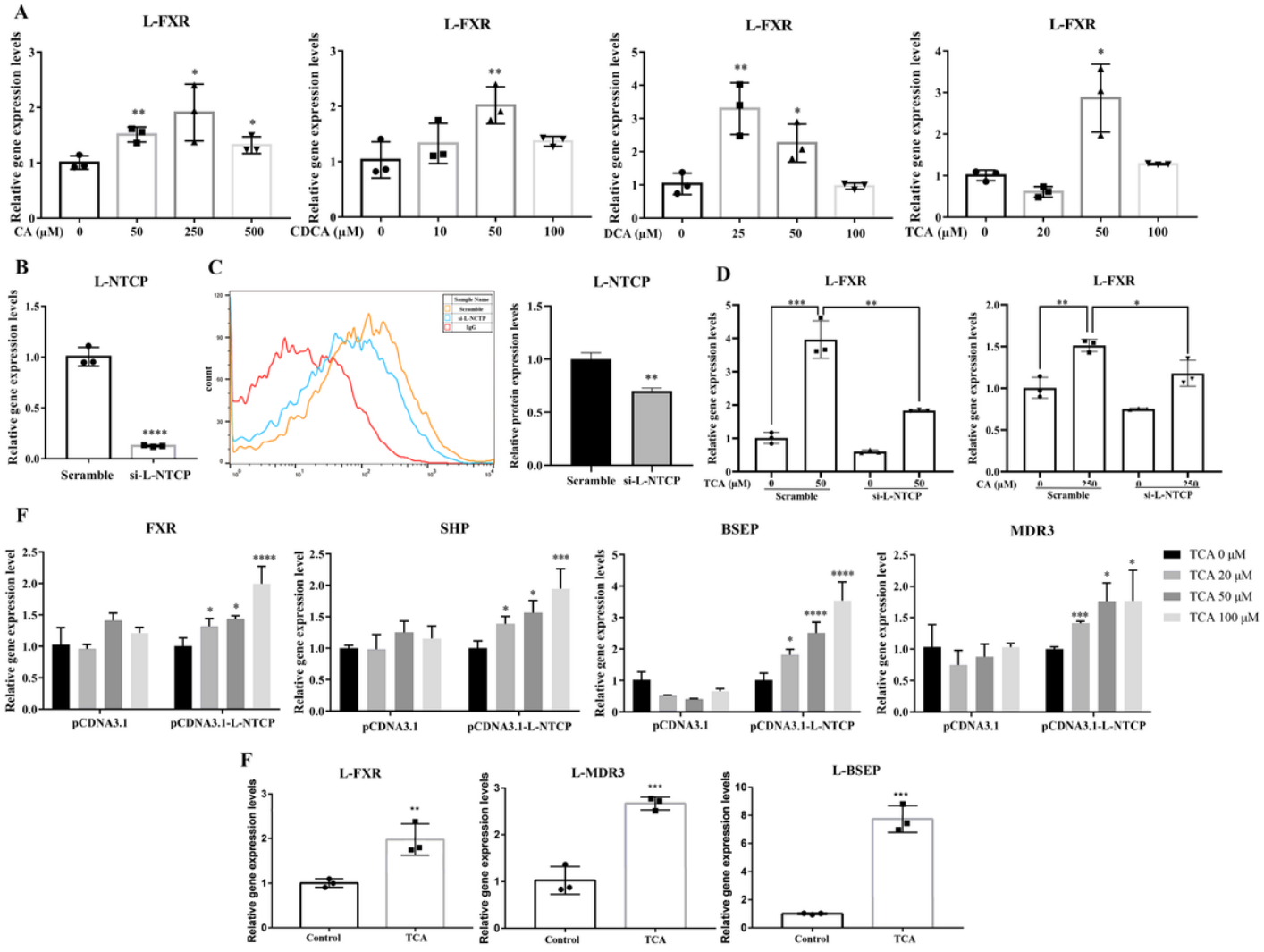

Figure 6

The biological function of L-NTCP is taking up bile acids. A. RT-qPCR detection of the relative mRNA expression levels of L-FXR in lamprey primary hepatocytes when treated with different concentrations of CA, CDCA, TCA and LCA for 24 hrs. B and C. Knock down of L-NTCP by siRNA. After transfection of siRNA-L-NTCP for 24hrs, RT-qPCR and flow cytometry was performed respectively to test the expression change of L-NTCP. D. RT-qPCR detection of the relative mRNA expression levels of L-FXR in lamprey primary hepatocytes. Following transfection of siRNA-L-NTCP for 24hrs, lamprey hepatocytes was treated with the indicated concentrations of CA or TCA for 24hrs. Cells were harvested and RT-qPCR was performed. Transfection of scrambled siRNA was as control. E. RT-qPCR detection of the relative mRNA expression levels of L-FXR in lamprey liver tissues. TCA was injected intraperitoneally at 10 mg/kg, and liver tissue samples were collected for the subsequent experiments. F. RT-qPCR detection of the relative mRNA expression levels of FXR in HepG2 cells. Following transfection of pcDNA3.1-L-NTCP or pcDNA3.1 for 24 hrs, HepG2 cells were treated with the indicated concentrations of TCA for 24 hrs before cell harvest.

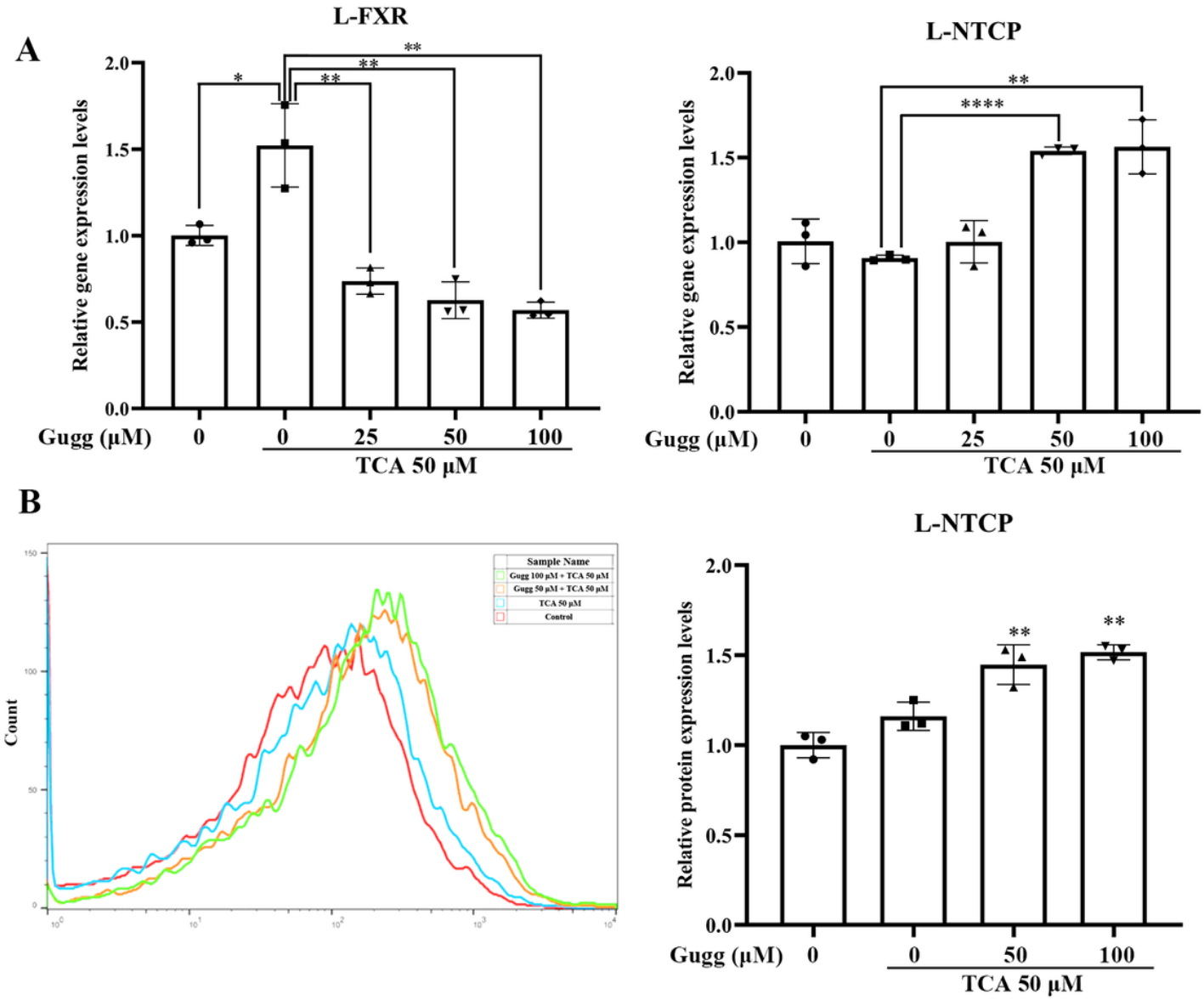

Figure 7

Expression regulation of L-NTCP by L-FXR. A. RT-qPCR detection of the relative mRNA expression levels of L-FXR and L-NTCP in lamprey primary hepatocytes. B. Flow cytometry detection of L-NTCP expression levels in lamprey primary hepatocytes. FXR antagonist guggulsterone was used to stimulate lamprey hepatocytes for 2hrs before the treatment of 50 μM TCA for 24hrs. Cells were harvested and performed by RT-qPCR and flow cytometry.

Supplementary Files

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