Role of TGR5 in Bile Acid Metabolism

APPROVED BY SUPERVISORY COMMITTEE

David J Mangelsdorf, Ph.D. (Mentor)

Steven A. Kliewer, Ph.D. (Mentor)

Carole R. Mendelson, Ph.D. (Chair)

David W. Russell, Ph.D.

Joyce J. Repa, Ph.D.

TO MY PARENTS

For your love and constant support

ACKNOWLEDGEMENTS

I must begin by thanking my mentors, Dr. David Mangelsdorf and Dr. Steven Kliewer. Their passion for science is true inspiration to me, and I am deeply grateful for the opportunity they gave me to grow in the Mango/Kliewer lab, as both a scientist and a person.

I would also like to acknowledge several people who inspired me to take the path as I did. First, Dr. Yi Zhang and her ribozyme laboratory gave me my first opportunity to experience life in a research lab. Dr. Philip Perlman changed the path of my life more than I had ever imagined by introducing me to UT Southwestern, and I am very grateful for that. I am fortunate to have the friendship of Carolyn Cummins and Elisabeth Martinez, who showed me how science and life should be experienced with hard work, dedication, love, and good laughter. Special thanks also go to the many current and former members of the Mango/Kliewer lab for their friendship over the years. The list includes Angie Bookout, Ewa Borowicz, Tara Ellison, Youn-Kyoung Lee, Zhu Wang, Yuan Zhang, Stan Atkin, Vicky Lin, and everyone else from A to XYZ.

I gratefully acknowledge the members of my thesis committee, Dr. Carole Mendelson, Dr. Joyce Repa, and Dr. David Russell for their thoughtful suggestions and guidance over the years. I thank Dr. Kris Kamm and her lab members for many helpful discussions and for sharing the very popular tensiometry machine. Thanks to Sam Holmstrom, Daniel Schmidt, Michi Umetani, Richard Hogg, and Robert Gerald for performing the miraculous surgeries on the bench that were essential to this project.

I thank my family and friends for their constant love and support. I thank my parents dearly for everything they have done and sacrificed for me. My words can never express the affection I feel for them. Thanks to my friends, Lina, Min, Wei, Nan, Fei, Beverly, Serena, Shelby, Xiao, Ke, and Bo for being the brothers and sisters I never had. Finally, I thank Jonathan. We have had to tackle some big challenges together, and I am always amazed how they actually made us stronger. Thank you for believing in me, for your unfailing support, and for being my best friend, teacher, and teammate.

ROLE OF TGR5 IN BILE ACID METABOLISM

by

TINGTING LI

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2010

ROLE OF TGR5 IN BILE ACID METABOLISM

TINGTING LI, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2010

David J. Mangelsdorf Ph.D., Steven A. Kliewer Ph.D.

TGR5 is a G protein-coupled bile acid receptor present in various tissues in the body. Its agonism increases energy expenditure and lowers blood glucose. Thus, it is an attractive drug target for treating human metabolic disease. However, TGR5 is highly expressed in the gallbladder, where its function is less well-characterized. In addition, *Tgr5*$^{-/-}$ mice are resistant to cholesterol gallstone disease (CGD), although the mechanism is poorly understood. Here, we demonstrate that TGR5 stimulates the filling of the gallbladder with bile. Gallbladder volume was increased in wild-type (WT) but not *Tgr5*$^{-/-}$ mice by administration of either the naturally-occurring TGR5 agonist, lithocholic acid (LCA), or the synthetic TGR5 agonist, INT-777. This effect did not require the presence

of fibroblast growth factor-15, an enteric hormone previously shown to stimulate gallbladder filling. *Ex vivo* analyses using gallbladder tissue showed that TGR5 activation increased cAMP concentrations and caused smooth muscle relaxation in a TGR5-dependent manner. These data reveal a novel, gallbladder-intrinsic mechanism for regulating gallbladder contractility. Further, a markedly decreased cholic acid/muricholic acid ratio was observed in *Tgr5*$^{-/-}$ mice, indicating increased hydrophobicity in the bile acid pool. Dysregulation of the expression of genes involved in bile acid transport were also observed. Our findings further suggest that TGR5 agonists should be assessed for effects on bile acid metabolism as these agonists are developed for treating metabolic disease. Potential mechanisms for TGR5 regulation of these different physiological and pathological processes are discussed.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Li T, Holmstrom RS, Kir S, Umetani M, Schmidt DR, Kliewer SA, Mangelsdorf DJ. The G Protein-Coupled Bile Acid Receptor, TGR5, Stimulates Gallbladder Filling. Submitted to *Mol Endo*.

Patel R, Patel M, Tsai R, Lin V, Bookout A, Zhang Y, Magomedova L, Li T, Chan J, Budd C, Mangelsdorf D, Cummins C. LXRβ is required for glucocorticoid-induced hyperglycemia and hepatosteatosis in mice. *J Clin Invest*. Epub 2010 Dec 1.

Katona BW, Cummins CL, Ferguson AD, Li T, Schmidt DR, Mangelsdorf DJ, Covey DF. Synthesis, characterization, and receptor interaction profiles of enantiomeric bile acids. *J Med Chem*. 2007 Nov 29;50(24):6048-58. Epub 2007 Oct 27.

Motola DL, Cummins CL, Rottiers V, Sharma KK, Li T, Li Y, Suino-Powell K, Xu HE, Auchus RJ, Antebi A, Mangelsdorf DJ. Identification of ligands for DAF-12 that govern dauer formation and reproduction in *C. elegans*. *Cell*. 2006 Mar 24;124(6):1209-23.

Xiao M, Li T, Yuan X, Shang Y, Wang F, Chen S, Zhang Y. A peripheral element assembles the compact core structure essential for group I intron self-splicing. *Nucleic Acids Res*. 2005 Aug 12;33(14):4602-11.

LIST OF FIGURES

LIST OF TABLES

LIST OF DEFINITIONS

Ad – Adventitia

AE2 – Anion exchanger 2

AF-1/2 – Activation function 1/2

ASBT – Apical sodium-dependent bile acid transporter (also see IBAT)

BAT – Brown adipose tissue

BSEP – Bile salt export pump (gene name ABCB11)

B.W. – Body weight

CA – Cholic acid

CCK – Cholecystokinin

CDCA – Chenodeoxycholic acid

CFTR – Cystic fibrosis transmembrane conductance regulator (gene name ABCC7)

CGD – Cholesterol Gallstone Disease

CYP27A1 – Sterol 27-hydroxylase

CYP7A1 – Cholesterol 7α-hydroxylase

CYP8B1 – Sterol 12α-hydroxylase

D2 – Type 2 iodothyroinine deiodinase

DCA – Deoxycholic acid

EGFR – Epidermal growth factor receptor

FGF – Fibroblast growth factor

FGFR4 – FGF receptor 4

FXR – Farnesoid X receptor (gene name NR1H4)

GLP-1 – Glucagon-like peptide 1

GPBAR-1 – G-protein-coupled bile acid receptor 1 (alternate name for TGR5)

GPCR – G-protein-coupled receptor

I-BABP – Ileal bile acid binding protein (gene name FABP6)

IBAT – Ileal bile acid transporter (synonym ASBT, gene name SLC10A2)

INT777 – 6α-ethyl-23(S)-methylcholic acid (S-EMCA, a TGR5 agonist)

LCA – Lithocholic acid

LRH1 – Liver receptor homolog-1 (gene name NR5A2)

LXR – Liver X receptor (gene name NR1H3 and NR1H2)

MAPK – Mitogen-activated protein kinase pathways

MCA – Muricholic acid

MDR3 – Multi-drug resistance protein 3 (gene name ABCB4)

ME – Muscularis Externa

mEH – Microsomal epoxide hydrolase

MRP2/MRP3 – Multidrug resistance-associated protein 2/3 (gene name ABCC2/ABCC3)

Mu – Mucosa

NHE – Na^+/H^+ exchanger

NTCP – Na^+-taurocholate cotransporting polypeptide (gene name SCL10A1)

OATP – Organic anion transporting protein (SLC01 gene family)

OSTα-OSTβ – Organic solute transporter

PKC – Protein kinase C

PPARs – Peroxisome proliferators-activated receptors (NR1C family)

PXR – Pregnane X receptor (gene name NR1I2)

SHP – Small heterodimer partner (gene name NR0B2)

SO – Sphincter of Oddi

SREBP1c – Sterol regulatory element binding protein 1c

SXR – Steroid and xenobiotic receptor (alternate name for PXR)

UDCA – Ursodeoxycholic acid

VDR – Vitamin D receptor (gene name NR1I1)

VIP – Vasoactive intestinal peptide

WT – Wild-type

CHAPTER I: INTRODUCTION

BILE ACIDS: AN OVERVIEW

Bile acids are cholesterol derivatives that are found mostly in bile and in the small intestine. The enzymatic conversion of cholesterol into bile acids in the liver, together with the excretion of cholesterol, is the most important means for the removal of cholesterol from the body. In an adult human, approximately 500 mg of cholesterol is converted into bile acids in the liver each day, with 16 or more enzymes involved in this transformation (Russell 2003). Newly synthesized bile acids are secreted into bile, stored in the gallbladder, and eventually emptied into the small intestine, where bile acids act as emulsifiers and promote the absorption of dietary lipids and fat-soluble vitamins. About 95% of secreted bile acids are recovered from the gut and returned to the liver through the portal vein during each cycle of enterohepatic circulation (Russell 2003). About 5% of the bile acid pool is excreted in the feces daily, and this amount is replenished in the liver by new synthesis. This synthetic process accounts for catabolizing 90% of the cholesterol that is actively metabolized every day (Russell 2003). Disturbances in bile acid synthesis have been shown to lead to hypercholesterolemia, atherosclerosis, gallstone disease, liver failure, and progressive neuropathy (Russell 2009).

Bile Acid Biosynthesis

The bile acid pool is composed of primary and secondary bile acids (Figure 1-1A). The primary bile acids in most mammalian species, including human and rat, are cholic acid (CA) and chenodeoxycholic acid (CDCA), which are produced in the liver

through multiple steps. This production is initiated by *Cyp7a1* (cholesterol 7α-hydroxylase) of the classical/neutral pathway or by *Cyp27a1* (sterol 27-hydroxylase) of the alternative/acidic pathway (Russell 2003). Sterol 12-α-hydroxylase (*Cyp8b1*) regulates the CA:CDCA ratio, which dictates the overall hydrophobicity of the bile acid pool. The primary bile acids are conjugated to glycine or taurine before being secreted from hepatocytes into the bile, which empties into the duodenum. In the intestine, primary bile acids are converted to secondary bile acids, e.g. deoxycholic acid (DCA) and lithocholic acid (LCA), by microbial enzymes. In mice, CDCA is efficiently converted into muricholic acid (MCA), and bile acids are almost exclusively conjugated to taurine (Russell 2003) (Figure 1-1A).

Because of the efficient recirculation of bile acids as mentioned earlier, only a small portion (5%) of the bile acid pool is synthesized *de novo*; however, this synthesis still occurs under multiple checkpoint regulation. The key enzymes and their regulation have been extensively studied and several reviews have described these pathways in detail (Repa and Mangelsdorf 1999; Chiang 2002; Russell 2003).

Function of Bile Acids

Apart from their essential role in regulating cholesterol metabolism, bile acids are needed for the absorption of dietary lipids and fat-soluble vitamins. Bile acids can facilitate the absorption of these hydrophobic substances due to the amphipathic nature of bile acids. Bile acids are molecules with a highly hydrophobic core with hydrophilic

hydroxyl groups attached (Figure 1-1A). Bile acids thus act like detergents to solubilize dietary lipids and promote their absorption in the intestine.

In addition, bile acids are signaling molecules. They bind nuclear receptors (Chiang 2002), activate mitogen-activated protein kinase (MAPK) pathways (Qiao, Studer *et al.* 2001), and are ligands for a G protein-coupled receptor (GPCR) termed TGR5 (Maruyama, Miyamoto *et al.* 2002; Kawamata, Fujii *et al.* 2003).

Nuclear Receptors

Nuclear receptors are ligand-activated transcription factors that regulate many genes involved in cell growth, differentiation, and metabolism. 48 nuclear receptor genes have been identified in the human genome. Nuclear receptors that have no identifiable ligand are referred to as orphan receptors. Some were eventually "adopted", including farnesoid X receptor (FXR), liver X receptor (LXR), and peroxisome proliferators-activated receptors (PPARs). Nuclear receptors have a modular structure with an N-terminal variable activation function 1 (AF1) domain, a highly conserved zinc finger DNA-binding domain, a hinge domain, a conserved ligand-binding domain, and a C-terminal activation function 2 (AF2) domain (Germain, Staels *et al.* 2006). In general, a nuclear receptor binds to co-repressor(s) in the absence of a ligand. Upon binding to a ligand, the nuclear receptor undergoes conformational changes and releases the corepressor(s), recruits a co-activator to the AF2 domain, and activates gene transcription.

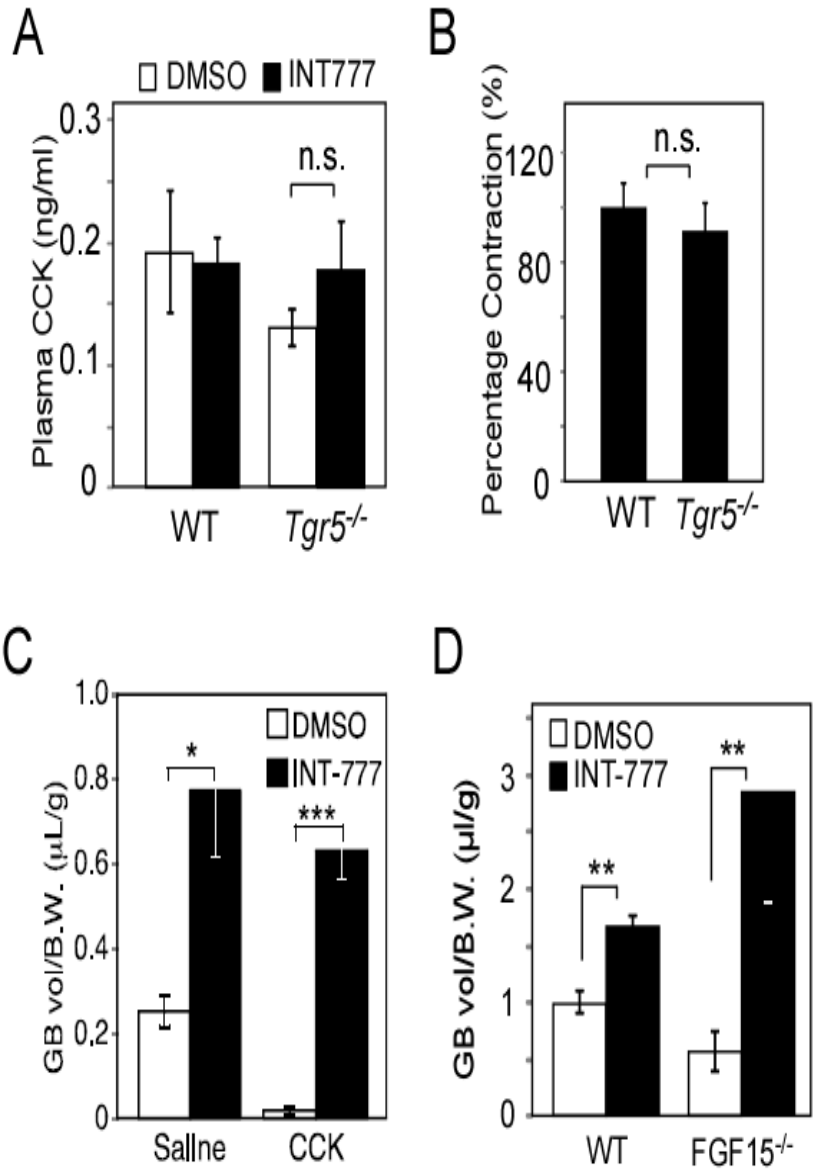


Figure 1-1. Bile acid structure and organic composition of bile.

A, Chemical structure of bile acids. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are primary bile acids. Deoxycholic acid (DCA) and lithocholic acid (LCA) are secondary bile acids. Muricholic acid (MCA) is a primary bile acid in rodents. **B**, Organic composition of (hepatic) bile. Percentage distribution (w/w) of bile acids (BAs), phospholipids (PL), cholesterol (Chol), proteins and bilirubin conjugates (BP). Figure adapted from Esteller (2008).

Many nuclear receptors have been found to play important roles in regulating transcription of the genes involved in bile acid synthesis. In general, hydrophobic bile acids (CA, CDCA, DCA and LCA) are potent inhibitors of bile acid biosynthesis, whereas hydrophilic bile acids, e.g. ursodeoxycholic acid (UDCA) and β-MCA are not (Repa and Mangelsdorf 1999). Three nuclear receptors have been identified as bile acid activated receptors: (1) FXR regulates multiple metabolic pathways, including bile acid synthesis, transport and absorption (Cai and Boyer 2006), reverse cholesterol transport, triglyceride synthesis, and glucose metabolism synthesis (Lu, Makishima *et al.* 2000; Chiang 2002; Watanabe, Houten *et al.* 2004; Ma, Saha *et al.* 2006). (2) Pregnane X receptor (PXR), or its human ortholog, steroid and xenobiotic receptor (SXR), can be activated by LCA and toxic bile acid derivatives, and regulates LCA and xenobiotic metabolism (Kliewer and Willson 2002; Goodwin, Gauthier *et al.* 2003). (3) Vitamin D receptor (VDR) can also be activated by LCA (Makishima, Lu *et al.* 2002), regulates bone and mineral homeostasis, and has a central role in calcium metabolism (Margolis and Christakos 2010).

Among the hydrophobic bile acids, CDCA is the most potent FXR ligand, with an EC_{50} of 50μM and 10μM *in vitro* on mouse and human FXR, respectively (Makishima, Okamoto *et al.* 1999). FXR is expressed in both the liver and the intestine. The activation of FXR inhibits bile acid biosynthesis and upregulates the excretion of bile acids, thus protecting enterohepatic tissues from the cytotoxic effects of bile acids. In the liver, FXR activation induces the expression of bile salt efflux pumps, leading to increased secretion of bile acids (Ananthanarayanan, Balasubramanian *et al.* 2001; Plass,

Mol *et al.* 2002). FXR also upregulates an atypical nuclear receptor, small heterodimer partner (SHP). SHP does not possess a DNA-binding domain, and it inhibits the activity of several nuclear receptors, including LXR and liver receptor homolog-1 (LRH-1), although the exact role of LRH-1 will need to be re-evaluated based on a recent study using the liver specific LRH-1 knockout mouse model (Lee, Schmidt *et al.* 2008). It had been believed that it is through the inhibition of LXR and LRH-1, that SHP mediates (1) the negative regulation of bile acid biosynthesis by through decreased expression of CYP7A1 (Goodwin, Jones *et al.* 2000; Lu, Makishima *et al.* 2000), and (2) the feedback regulation of hepatic fatty acid and triglyceride biosynthesis by repressing the transcription of sterol regulatory element-binding protein 1c (SREBP1c) (Watanabe, Houten *et al.* 2004).

In the intestine, the activation of FXR regulates bile acid metabolism by inducing the expression of a protective ileum bile acid binding protein (I-BABP) and the fibroblast growth factor 19 (FGF19, mouse ortholog FGF15). I-BABP is a soluble bile acid binding protein located in ileocytes. It protects ileocytes from the toxic effects of bile acids and may be critical for the enterohepatic circulation of bile acids (Landrier, Grober *et al.* 2002). FGF-19 is a secreted hormone that travels to the liver and activates fibroblast growth factor receptor 4 (FGFR4) to ultimately inhibit CYP7A1; this inhibition is SHP-dependent (Holt, Luo *et al.* 2003; Inagaki, Choi *et al.* 2005). FGF19 also has an effect on gallbladder motility, which will be discussed later in this chapter. It is worth noting that transgenic mice overexpressing FGF19 display increased energy expenditure and decreased adiposity; they also are resistant to diet-induced obesity and diabetes. This

phenotype is attributed to increased brown adipose tissue (BAT) mass and/or increased fatty acid oxidation due to decreased ACC2 expression (Tomlinson, Fu *et al.* 2002).

Further, SHP has been reported to inhibit PGC1α expression and energy production in BAT (Wang, Liu *et al.* 2005). These authors report that mice lacking SHP had upregulated PGC1α expression, increased energy expenditure, and resistance to diet-induced obesity. It is unclear whether this effect is directly linked to the absence of SHP. Further research will be needed to dissect the role of SHP *per se*, the increased bile acids pool, and the altered bile acid homeostasis found in these animals.

TGR5: A Novel Bile Acid Receptor

The effect of bile acids on energy and glucose metabolism has attracted much attention due to the recent discovery of a novel G protein-coupled receptor, TGR5 (also named GPBAR-1, M-BAR and BG37). TGR5 is Gs-coupled and activates cAMP production upon bile acid treatment (Maruyama, Miyamoto *et al.* 2002; Kawamata, Fujii *et al.* 2003). It is activated by bile acids in a dose-dependent manner when measuring cAMP production in CHO-hTGR5 cells treated with bile acids, with EC_{50} of: TLCA (0.33 μM) < LCA (0.53 μM) < DCA (1.01 μM) < CDCA (4.43μM) < CA (7.72 μM). Bile acid activity on TGR5 seems to increase in accordance with the hydrophobicity of the ligand (Kawamata, Fujii *et al.* 2003). In contrast, FXR has CDCA as by far the most efficacious ligand (Makishima, Okamoto *et al.* 1999). Further, unconjugated and conjugated bile acids are all active TGR5 agonists (Kawamata, Fujii *et al.* 2003).

The mRNA of TGR5 has a very interesting expression profile. It has been detected in various tissues in humans, rats, mice, and rabbits. In mice, it is found in the gallbladder, gastrointestinal tract, ovary, placenta, and testis, but it is expressed in the gallbladder at levels 60-100 times higher than any of these other tissues (Vassileva, Golovko *et al.* 2006). In humans, it has been reported to be expressed in the kidney, spleen, adipose tissue, uterus, ovary and mammary gland in addition to the enterohepatic tract (Kawamata, Fujii *et al.* 2003). In addition to evidence supporting a role in obesity, diabetes, and the hepatobiliary system, which I will discuss in detail later, TGR5 is detected in monocytes and macrophages, and activation of TGR5 inhibits the expression of inflammatory cytokines in activated macrophages (Kawamata, Fujii *et al.* 2003; Keitel, Donner *et al.* 2008).

It has been reported that a diet supplement of 0.5% CA protects mice from diet-induced obesity (Ikemoto, Takahashi *et al.* 1997). Watanabe *et al.* (2006) report similar findings; they also show that administration of bile acids leads to increased energy expenditure in BAT and that the bile acid induced energy expenditure is dependent on the induction of the cAMP-dependent type 2 iodothyroinine deiodinase (D2), as suggested by the loss of bile acid-mediated protective effect in $D2^{-/-}$ mice. D2 is the enzyme that activates the conversion of thyroxine (T4) into 3,5,3'-triiodothyronine (T3). Thus, this report supports the idea that local regulation of thyroid hormone activity can be involved in metabolism and that this pathway plays a much broader role than previously believed (Bianco and Kim 2006). This activation of D2 is FXR-independent, and the involvement

of TGR5 is suggested based on its co-localization with D2 in BAT and skeletal muscle and data from *in vitro* reporter assays (Watanabe, Houten *et al.* 2006).

The possibility of a role of TGR5 in peripheral tissues is supported by the fact that the physiological range of serum bile acid concentration correlates well with their EC_{50} for TGR5 (0.3-10 μM). Bile acids are found between 0.1-1.6μM in fasting conditions, up to 3.6μM after feeding (Angelin, Bjorkhem *et al.* 1982), and around 20 μM in portal blood (Dancygier 2009). However, in a recent study, a few (patho)physiological conditions were analyzed in humans, and the level of total bile acids or individual bile acid species did not show any correlation with energy expenditure (Brufau, Bahr *et al.* 2010). In addition, TGR5 mRNA is expressed at relatively low levels in BAT and skeletal muscle, according to previous reports conducted in mice and humans (Kawamata, Fujii *et al.* 2003; Vassileva, Golovko *et al.* 2006), and it is still controversial whether BAT contributes to metabolism in human adults. Thus, the physiological significance of TGR5-D2 connection in BAT will need to be further evaluated.

TGR5 has also been shown to activate the production of glucagon-like peptide 1 (GLP-1). *In vitro* data for TGR5 induced GLP-1 secretion has been presented in an enteroendocrine cell line, STC-1 (Katsuma, Hirasawa *et al.* 2005), and the potential role for TGR5 was further supported when a higher level of GLP-1 was also detected in plasma when the TGR5 agonist was administered to mice *in vivo* (Thomas, Gioiello *et al.* 2009). In addition, a natural TGR5 agonist from olive leaves (*O. europaea*) decreased plasma glucose and insulin levels in C57BL/6J mice fed on a high-fat diet for 10 weeks and then given a 7-day treatment of oleanolic acid (Sato, Genet *et al.* 2007).

The involvement of TGR5 in regulating GLP-1 secretion is important because the latter is involved in many aspects of diabetes and obesity. GLP-1 has important roles in the pancreas as shown by its stimulation of glucose dependent insulin secretion in islets and its inhibition of glucagon secretion in the pancreas (reviewed by Drucker 2006). In fact, GLP-1 based therapy has proven to be effective in treating type 2 diabetes (Brubaker 2010). The extrapancreatic roles of GLP-1 include regulation of hepatic glucose production, inhibition of gastric emptying and gastric acid secretion, increasing satiety and decreasing appetite, as well as cardioprotective and cardiotropic effects (reviewed by Abu-Hamdah, Rabiee *et al.* 2009). For these reasons, TGR5 is considered a promising target in the therapeutic management of diet-induced obesity and diabetes.

Analyses using *Tgr5*$^{-/-}$ mice also reveal a role for TGR5 in bile acid metabolism. Maruyama and colleagues (2006) have shown that the *Tgr5*$^{-/-}$ mice have a smaller bile acid pool size and that female knockout mice gain more body weight when placed on high-fat diet for 2 months. TGR5 has been linked to gallstone formation in mice, with *Tgr5*$^{-/-}$ mice protected from cholesterol gallstone disease when fed a lithogenic diet (Vassileva, Golovko *et al.* 2006). In addition, TGR5 protein is detected in liver sinusoidal endothelium and Kupffer cells (Keitel, Reinehr *et al.* 2007; Keitel, Donner *et al.* 2008; Keitel, Cupisti *et al.* 2009). TGR5 protein is co-localized with CFTR, and activation of TGR5 increases chloride secretion *in vitro* (Keitel, Cupisti *et al.* 2009). While no genetic variation within TGR5 has been identified in patients with increased risks for type 2 diabetes (Mussig, Staiger *et al.* 2009), six nonsynonymous mutations have been found in patients with primary sclerosing cholangitis (PSC), a chronic inflammatory bile duct

disease, and five out of the six mutations reduce or abolish TGR5 function in an activity assay *in vitro,* suggesting a role of TGR5 and bile acids in intestinal and biliary inflammation (Hov, Keitel *et al.* 2010).

I became interested in the function of TGR5 in the gallbladder in part because of its strikingly high expression level in the gallbladder (our data; Vassileva, Golovko *et al.* 2006; Keitel, Cupisti *et al.* 2009). My analysis of *TGR5*$^{-/-}$ mice revealed a striking phenotype in gallbladder motility of *TGR5*$^{-/-}$ mice. At the time of preparing this manuscript, Lavoie and colleagues (2010) also reported a role of TGR5 in gallbladder smooth muscle. I will present both *in vitro* and *in vivo* data analyzing the role of TGR5 in the gallbladder and other hepatobiliary tissues. I will discuss our understanding in the context of evidence presented by Lavoie *et al.*

Nonspecific Activities of Bile Acids

Apart from direct activation of *bona fide* bile acid receptors, e.g., FXR and TGR5, it is believed that bile acids can activate cellular responses by activating other types of receptors. Both conjugated and unconjugated bile acids have been shown to induce transactivation of epidermal growth factor receptor (EGFR) in hepatocytes, intestinal epithelial cells, and colon cancer cells, although it is controversial as to whether this activation is EGF-dependent or not (Qiao, Studer *et al.* 2001; Cheng and Raufman 2005; Merchant, Rogers *et al.* 2005). Interestingly, a role for TGR5 has more recently been suggested by a study conducted in AGS cells, a gastric carcinoma cell line, in which the receptor tyrosine phosphorylation of EGFR was strongly suppressed by RNAi knockout of TGR5 (Yasuda, Hirata *et al.* 2007).

Finally, bile acids can elicit cellular responses by perturbing membrane structure and altering membrane microdomains (Jean-Louis, Akare *et al.* 2006). Due to their detergent properties, hydrophobic bile acids can be incorporated into membrane micro-domains, decrease membrane fluidity, and increase membrane cholesterol content. It has been shown that a high concentration of DCA (500 μM) causes redistribution of scaffolding proteins in cell membranes. At this concentration, DCA was also found to induce tyrosine phosphorylation and activate the receptor tyrosine kinase activity of EGFR, albeit in a ligand-independent manner (Jean-Louis, Akare *et al.* 2006).

These activities of bile acids might lead to a host of different downstream cellular responses, adding to the complexities of the bile acid-induced signaling network. It is worth noting that each of these pathways is activated by bile acids at unique rank order potency. Thus, perturbations in concentration or composition of the bile acid pool could have profound effects on each of the above mentioned pathways.

Physiology and Pathology of Bile Secretion: an Overview

The secretion of bile is important for normal digestive function and is the primary means by which the body eliminates hydrophobic molecules, including cholesterol and xenobiotics (Forker 1977). Bile is a complex fluid composed of water, electrolytes and a mix of various organic molecules including conjugated bile acids, cholesterol, phospholipids, bilirubin and conjugated steroid hormones (Toouli and Bhandari 2007). Figure 1-1B illustrates the major organic constituents of bile. Bile acids being the predominant organic constituents of bile are circulated between the liver and

intestine. Another major component of bile, phospholipids, form micelles with bile acids and are crucial for the solubilization of cholesterol as well as other hydrophobic compounds (Meier and Stieger 2000).

The secretion of bile is an important function of the liver. The biliary apparatus, which provides the structural basis for hepatic bile secretion, is an organized system of canals that begins with the canaliculi, continues with the bile ducts, and ends with the common bile duct (Tooull and Bhandari 2007). Hepatocytes, which account for about 65% of the liver cell population, secrete most of the lipid contents to form the primary bile at the canalicular membrane (Esteller 2008). This process is dependent on the ATP binding cassette (ABC) transporters located on the canalicular membrane (Wang, Cohen *et al.* 2009). In short, bile salt export pump (BSEP or ABCB11) mediates the secretion of bile acids (or more accurately, bile salts) (Gerloff, Stieger *et al.* 1998; Wang, Salem *et al.* 2001); multi-drug resistance protein (MDR3 or ABCB4) governs the secretion of phospholipids (Smit, Schinkel *et al.* 1993); and ABCG5/ABCG8 mediates the secretion of cholesterol (Yu, Li-Hawkins *et al.* 2002; Wang, Patel *et al.* 2007). While FXR upregulates the expression of BSEP and MDR3, LXR regulates the expression of ABCG5/ABCG8 (Kalaany and Mangelsdorf 2006).

Primary canalicular bile enters the bile duct and is further modified by bile ductal epithelial cells, cholangiocytes. Cholangiocytes dilute and alkalinize the primary bile by absorbing electrolytes and secreting fluid and bicarbonate in response to various signals from hormones such as secretin, glucagon, somatostatin, insulin and gastrin (Strazzabosco, Fabris *et al.* 2005). It is believed that these signals converge on cAMP

formation, which activates the phosphorylation of the cystic fibrosis (CF) transmembrane conductance regulator (CFTR), ultimately stimulating Cl^- and HCO_3^- efflux and inhibiting NHE3-mediated Na+ absorption (Cohn, Strong *et al.* 1993; Mennone, Biemesderfer *et al.* 2001). The sodium-independent Cl^-/HCO_3^- anion exchanger (AE2) is expressed in the apical membrane of cholangiocytes (Martinez-Anso, Castillo *et al.* 1994) and coordinates with CFTR in the excretion of HCO_3^-. Water is then secreted following the osmotic gradient, both paracellularly and through water channels, e.g. aquaporin 1 and 4 (AQP1 and AQP4) (Marinelli, Tietz *et al.* 1999; Marinelli, Pham *et al.* 2000). Cholangiocytes also absorb biliary constituents such as glucose and bile acids (Strazzabosco, Fabris *et al.* 2005). The latter contribute to cholehepatic recirculation of bile acids and may be important for the overall regulation of bile secretion (Hofmann 2007). While bile acids have been shown to enhance cholangiocyte proliferation (Alpini, Glaser *et al.* 1997), excess bile acids can damage the epithelial barrier function and lead to inflammatory responses (Fickert, Fuchsbichler *et al.* 2004). In addition, cholangiocytes participate actively in immune functions, proliferation and differentiation in development and during liver regeneration (Adams 1996; Strazzabosco 1997). Notably, CFTR is expressed only in cholangiocytes, not hepatocytes (Cohn, Strong *et al.* 1993). A defect in this cAMP-dependent Cl^- channel as well as other transporters located in cholangiocytes can lead to impaired bile ductal secretion in cholangiocytes and eventually CF liver disease and cholestasis (Cho 2000).

Another disease related to bile physiology is cholesterol gallstone disease. Cholesterol crystallization is a result of cholesterol exceeding the solubilizing capacity of

the phospholipid-bile acid micelles, while excess bile acids in relation to phospholipids lead to lowered cholesterol solubilizing capacity of the bile (Carey 1978; Wang and Carey 1996; Wang, Cohen *et al.* 2009). Cholesterol supersaturation, hydrophobic bile acids, pronucleating proteins and impaired gallbladder motility are contributing factors for the pathogenesis of gallstones (van Erpecum 2005; Wang, Cohen *et al.* 2009). Changes in the FXR and/or LXR activity may have profound effects on the biliary lipid secretion by the liver. In fact, Moschetta *et al.* observed in $Fxr^{-/-}$ mice, increased cholesterol precipitation, increased bile salt hydrophobicity and gallbladder inflammation, while treatment with an FXR agonist in WT mice upregulated BSEP and MDR3 mRNA levels and protected the animals from gallstone formation (Moschetta, Bookout *et al.* 2004).

THE GALLBLADDER: AN OVERVIEW

Most vertebrates possess a gallbladder (exceptions include the rat, bird and horse) (Romer and Parsons 1977). The gallbladder is considered a non-essential organ as it can be removed without significant consequences. However, the gallbladder is an integral part of the gastrointestinal system and has important digestive functions. In addition, gallbladder disease is a frequent problem in developed countries and one of the most expensive digestive diseases in the United States, costing over $6 billion in health care expenditures each year (Everhart and Ruhl 2009). The primary function of the gallbladder is to store and concentrate the bile. The contractile or motor function of the gallbladder and its regulation of hydrostatic pressure in the biliary tract are important for its function as the physical pump, while the gallbladder epithelium modifies the

composition of bile in the gallbladder, adding to the role of this remarkable organ in the normal digestive processes in the body (Barrett, Ghishan *et al.* 2006).

Although the full scope of gallbladder function and physiology is still not well understood, the gallbladder has been extensively studied, in large part due to the prevalence and clinical significance of gallbladder disease. Also, the gallbladder epithelium has long served as a model for leaky membranes because of the parallels found between transport regulation in the gallbladder and in epithelium of "greater homeostatic significance" (Reuss, Segal *et al.* 1991; Barrett, Ghishan *et al.* 2006).

The functions of the gallbladder are reflected in its microanatomic structure. The wall of the gallbladder contains a mucosa (Mu), a muscularis externa (ME), and an adventitia (Ad) (Hopwood and Ross 1997). The Mucosa is extensively folded and consists of a single layer of columnar epithelium that lies atop a well-developed lamina propria made of loose connective tissue. The muscularis externa consists of smooth muscle fibers in both circular and longitudinal orientations. The muscularis externa is surrounded by an envelope of connective tissue, the adventitia, which also contains vessels and nerves surrounding the gallbladder. Each of these different structural components has a unique role.

Gallbladder Mucosal Function

Bile is synthesized and secreted at considerable metabolic expense in the hepatocytes, and more than half of the hepatic bile is taken up by the gallbladder, where bile is concentrated during fasting to 10-20% of its original volume, via additional

energy-consuming processes (Davenport 1966). It is estimated that the concentration process of bile across the gallbladder epithelium occurs against a concentration gradient of about 10,000:1 (Klinkspoor and Lee 2000). The gallbladder Mucosa, or more specifically, the gallbladder epithelium, is responsible for ensuring the directional/vectorial transport of water, electrolytes and macromolecules across the epithelial membrane. The epithelial cells are simple, tall, and polarized cells, with an asymmetrical distribution of their intracellular organelles and polarized organization of their plasma membrane (Hopwood and Ross 1997). The apical membrane of the epithelial cells faces the lumen and has microvilli, and the basolateral membrane has a basal region that is anchored to the basement membrane. The two membrane domains are separated by tight junctions that provide a selective permeability barrier and prevent water-soluble molecules from leaking between the epithelial cells (Hopwood and Ross 1997). The subepithelial layer of the gallbladder contains connective tissue, smooth muscle, blood vessels, and serosa. The transport across the epithelium occurs to or from the underlying capillaries, and the blood compartment in the subepithelial layer functions as a sink for the transported water and electrolytes (Lee 2000).

Gallbladder Electrolyte and Water Transport

The gallbladder concentrates bile by absorbing water and NaCl in near-isosmotic proportions (Diamond 1964). There is still controversy regarding the precise mechanism underlying this function, but recent studies support an electroneutral, double Na^+/H^+, $Cl^-/HCO3^-$ exchange model (Figure 1-2) (Lee 2000).

The NHE3 isoform of the Na^+/H^+ exchanger on the apical membrane is believed to mediate sodium uptake, and basolateral membrane Na^+ exit is mediated by the Na^+/K^+ activated ATPase (Figure 1-2) (Reuss, Segal *et al.* 1991). It has been shown that the AE2 Cl^-/HCO_3^- exchanger is present in the apical membrane of gallbladder epithelial cells and is thought to be involved in the bicarbonate secretion into bile (Figure 1-2) (Scoazec, Bringuier *et al.* 1997). The basolateral membrane Cl^- transport from cell to basolateral fluid is thought to result from both conductive transport and electroneutral KCl cotransport (Reuss, Segal *et al.* 1991).

The absorption of water, on the other hand, is thought to occur via a passive process coupled to the salt transport, even though it might be against the electrochemical gradient (Reuss 1991). It is believed that salt transport causes small osmotic gradient across both sides of the gallbladder epithelial membrane, making the cell interior hyperosmotic to the lumen and hypoosmotic to the extracellular fluid lateral of the epithelial cells, thus removing water from the bile and concentrating it in the gallbladder.

Regulation of Electrolyte And Water Transport

Transepithelial electrolyte absorption is the result of the integrated effects of transporters involved in this process. The activity of ion transporters, and thus the rate of water absorption, are modulated by factors involving membrane voltage, covalent modification (e.g. phosphorylation, methylation), and alterations in the membrane density of these transporters (Reuss, Segal *et al.* 1991). In the gallbladder epithelium, the best understood regulatory mechanisms involve intracellular factors, including pH, cAMP,

and Ca^{2+}. Changes in intracellular concentrations of these agents modulate the effects of hormones and neurotransmitters on the ion transport. For example, prostaglandins, secretin, and vasoactive intestinal peptide (VIP) inhibit fluid absorption by the gallbladder, while CCK has no effect (Wood and Svanvik 1983; O'Grady, Wolters *et al.* 1989; Klinkspoor and Lee 2000). The effect of cAMP on gallbladder epithelial cells is reviewed by Reuss, Segal, and Alternberg (1991). Elevating intracellular cAMP concentration has inhibitory effects on fluid absorption by the gallbladder (in some cases, even induces net secretion), and the dominant effect of cAMP is exerted on the apical membrane by activation and/or insertion of Cl^- channels (e.g. CFTR) and inhibition of both Na^+/H^+ and Cl^-/HCO_3^- exchangers (Petersen and Reuss 1983; Petersen, Wehner *et al.* 1985; Reuss 1987). Because prostaglandins, secretin, and VIP can elevate intracellular cAMP levels, it is suggested that cAMP can mediate the inhibitory effect of these agents (Reuss, Segal *et al.* 1991). The effect of cAMP on the electrolyte transport function of the gallbladder is one of the reasons that led me to perform preliminary tests on the electrolyte function of the gallbladders of *Tgr5*$^{-/-}$ mice (data not shown).

Gallbladder Secretion

Although the main function of the gallbladder epithelium is to absorb water and electrolytes, gallbladder mucosa also secretes mucins, which are glycoproteins composed of approximately 15-20% protein and 80% carbohydrate (Strous and Dekker 1992;

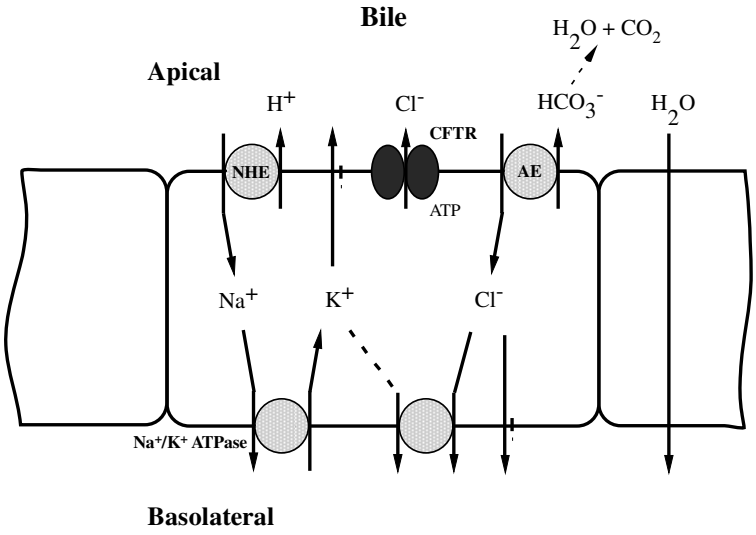


Figure 1-2. Ion transport model for gallbladder epithelium.

Figure depicts directions of fluxes observed in steady state. NaCl enters from the apical membrane via parallel Na^+/H^+ exchanger (NHE) and Cl^-/HCO_3^- anion exchanger (AE). Basolateral Na^+ extrusion is mediated by the Na^+/K^+-ATPase pump, and Cl^- via KCl cotransport and a Cl^- conductive pathway. H^+ and HCO_3^- are transported to the lumen as a result. Part of the luminal HCO_3^- dissociates as CO_2 and H_2O. Water follows the osmotic gradient created by the sodium flow and is absorbed by the subepithelial capillaries. Figure adapted from Reuss *et al* (1991).

Offner 2000). These glycoproteins form multimers which can result in a molecular weight of more than 10,000 kDa. The hydrophilic mucin multimers expand quickly in the presence of water and form a protective mucous layer lining the gallbladder epithelium (Sheehan, Oates *et al.* 1986; Sellers, Allen *et al.* 1988). Thus under normal conditions,

the mucous layer formed by mucins provides a barrier between the epithelial cells and the high concentrations of bile acids and phospholipids in the bile. However, mucins can play a major role in the pathogenesis of cholesterol gallstone disease, and when hypersecreted, can pose serious concerns. Mucin hypersecretion precedes gallstone formation (Lee, LaMont *et al.* 1981); the glycoproteins can serve as the nucleation core for gallstone formation (Maki, Matsushiro *et al.* 1971; Smith 1987; de la Porte, Domingo *et al.* 1996). Overexpression *MUC1* in C57BL/6J mice increased the susceptibility of these mice to gallstone formation (Wang, Afdhal *et al.* 2006). Mice lacking *MUC1,* one of the mucin encoding genes, are resistant to gallstone formation on a lithogenic diet (Wang, Afdhal *et al.* 2004). Further, multiple polymorphisms in three different mucin genes are associated with gallstone disease in men (Chuang, Juo *et al.* 2010).

Regulation of Mucin Secretion

The secretion of mucins has been studied primarily in relation to gallstone disease. The mediator and intracellular signals that lead to increased mucin secretion are unclear, but there are several potential mechanisms.

In prairie dogs, the cholesterol-induced hypersecretion of mucins is prevented by the ligation of the cystic duct (Lee, LaMont *et al.* 1981), suggesting that a substance stimulating mucin secretion is carried into the gallbladder by hepatic bile. Studies carried out in ground squirrels suggest that mucin hypersecretion occurred within 18 hours of cholesterol feeding and lasted throughout the 20-week experimental period (Pemsingh, MacPherson *et al.* 1987; MacPherson and Pemsingh 1997). Among the substances that

have been shown to stimulate mucin secretion are PKC, Ca^{2+}, forskolin, arachidonic acid, prostaglandins, phospholipids, and bile acids (LaMont, Turner *et al.* 1983; LaMorte, LaMont *et al.* 1986; McCool, Marcon *et al.* 1990; Forstner, Zhang *et al.* 1993; Forstner, Zhang *et al.* 1994; Klinkspoor, Kuver *et al.* 1995).

Bile acids have been shown to stimulate mucin secretion in the gallbladder and the colon, but mechanistic studies have not yielded clear results (Klinkspoor, Kuver *et al.* 1995; Klinkspoor, Tytgat *et al.* 1996; Shekels, Lyftogt *et al.* 1996; Klinkspoor, Mok *et al.* 1999). Among the bile acids tested, hydrophobic bile acids (DCA, CDCA), but not the more hydrophilic bile acids (CA, UDCA), are potent stimulators for mucin secretion in primary epithelial cells from dogs (Klinkspoor, Kuver *et al.* 1995) as well as in human colon cell lines including differentiated Caco2 and HT29 cells (Shekels, Lyftogt *et al.* 1996; Klinkspoor, Mok *et al.* 1999), with measurable differences detected within 30 minutes.

Enterohepatic Circulation of Bile Acids

The enterohepatic circulation of bile acids is driven by two physical pumps (gallbladder and intestine) and two chemical pumps (active bile acid uptake by the ileum and by the liver) (Northfield, Ahmed *et al.* 2000). The chemical pumps are very efficient in transporting bile acids, and bile acids thus circulate rapidly through the enterohepatic system when a person or animal eats, which stimulates the physical pumps to eject bile acids that reach the intestinal site of absorption (Lanzini and Lanzarotto 2000).

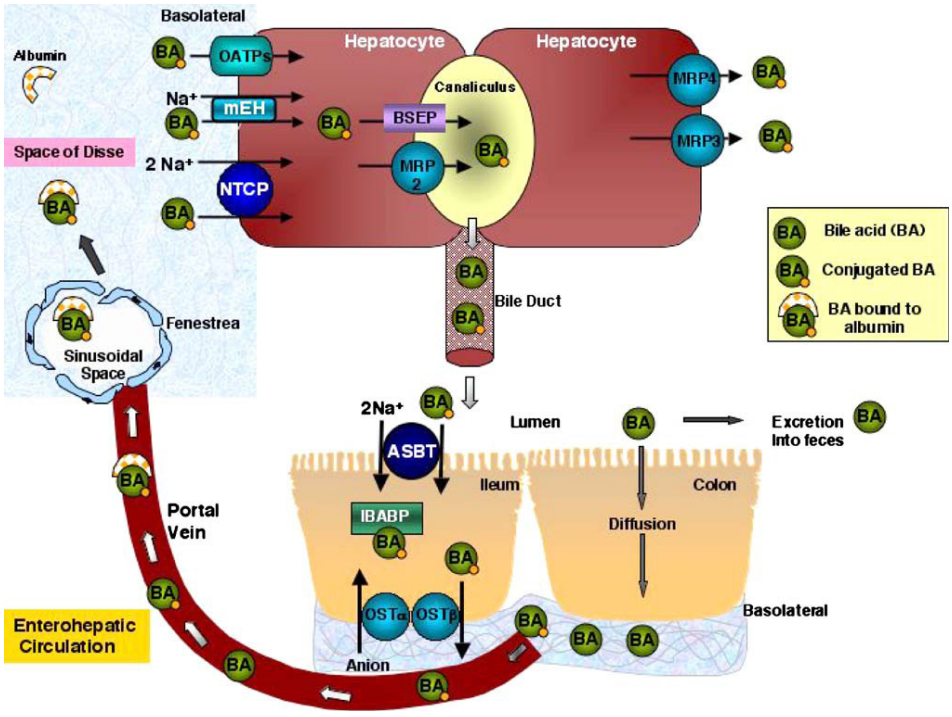


Figure 1-3. Enterohepatic circulation of bile acids.

The chemical pumps at each step are: NTCP (as well as mEH and OATP) for uptake of bile acids into the hepatocytes at the basolateral membrane, while MRP3 and MRP4 mediate bile acid efflux across the basolateral membrane; BSEP and MRP2 for the secretion of bile acids across the canalicular membrane; ASBT for active absorption of bile acids in the distal ileum, with I-BABP mediating the intracellular transport of bile acids across the enterocytes, and lastly, OST α /OST β for bile acid efflux from enterocytes. Bile acids return to the liver via the portal circulation and complete the enterohepatic circulation. Reprinted from Alrefai and Gill (2007). Used with permission from Springer Science+Business Media and the original authors.

Bile Acid Transporters: The Chemical Pumps

Chemical pumps are essential for circulating bile acids in the intestine and the liver. The function and regulation of the major bile acid transporters has been extensively reviewed (Hoffman 1994; Alrefai and Gill 2007). In the liver, bile acids are extracted from the portal blood against a concentration gradient across the basolateral membrane of the hepatocytes. The portal blood supplies its content, including bile acids, through large pores (fenestrae) of the sinusoids, which are in direct contact with the hepatocyte (Meier and Stieger 2000). This process constitutes the first step of the portal clearance of bile acids into the hepatocytes and is mediated by the Na(+) taurocholate cotransporting polypeptide (NTCP) with assistance from the organic anion transporting proteins (OATPs) (Figure 1-3). The bile acids are then secreted in an ATP-dependent fashion across the canalicular membrane by the bile salt export pump (BSEP) and multidrug resistance protein 3 (MRP3) into the bile, also against a concentration gradient (Figure 1-3). Following their movement with bile, bile acids travel through bile ducts and the gallbladder into the lumen of the small intestine and are efficiently reabsorbed in the ileum by the apical sodium-dependent bile acid transporter (ASBT). At the enterocyte basolateral membrane, bile acids are effluxed into the portal circulation by the heterodimeric organic solute transporter, OSTα-OSTβ (Figure 1-3).

Although the majority of bile acids are reabsorbed in the ileum, in the more proximal jejunum, absorption of conjugated bile acids is driven by anion exchange in the brush-border membrane vesicles (Amelsberg, Jochims *et al.* 1999). In the small intestinal and colonic epithelia, unconjugated bile acids can also passively diffuse to a degree (St-Pierre, Kullak-Ublick *et al.* 2001). In addition, subgroups of these bile acid transporters

are expressed in the biliary, renal, and colonic epithelium (Dawson, Lan *et al.* 2009). With the facilitation of these transporters, bile acids are absorbed in the intestine and return to the liver via the portal circulation. This completes the enterohepatic circulation of bile acids (Alrefai and Gill 2007).

Gallbladder: The Physical Pump

Bile is stored in the gallbladder during interdigestive periods, until the ingestion of food activates cholecystokinin (CCK) and stimulates gallbladder emptying. The filling and emptying of the gallbladder is determined by various neural, hormonal, and paracrine factors (Jazrawi 2000; Portincasa, Di Ciaula *et al.* 2008). Interdigestive motility may be more important in the pathogenesis of gallstone disease (Van Erpecum and Van Berge-Henegouwen 1999).

Regulation of Gallbladder Motility: Neural Control

The gallbladder is innervated both extrinsically by parasympathetic and sympathetic nerves and intrinsically by intramural plexi involving cholinergic, catecholinergic, serotonergic, and peptinergic neurons (Jazrawi 2000). The central nervous systems may modulate the enteric neural circuits that regulate the digestive state. Cholinergic (parasympathetic) stimulation through the vagus nerves is considered the major neural factor regulating gallbladder motility. Vagal activity might contribute to resting gallbladder tone because vagotomy results in gallbladder dilatation (Amdrup and Griffith 1970), while cholinergic blockage with atropine has no effect on the resting tone (Hopman, Jansen *et al.* 1987). There is also evidence suggesting that vagal stimulation is

involved in gallbladder response to sham feeding (Fisher, Rock *et al.* 1986; Yamamura, Takahashi *et al.* 1988). In addition, during the interdigestive period, the gallbladder periodically contracts, resulting in emptying of up to 30% of that seen after a meal (Marzio, Neri *et al.* 1988). This periodical contraction is almost abolished by atropine, suggesting that cholinergic activity is responsible for this interdigestive emptying (Svenberg, Christofides *et al.* 1982). The role of the sympathetic nervous system in gallbladder motility is unclear.

Regulation of Gallbladder Motility: Hormonal Control

The endocrine control of gallbladder motility involves both intrinsic and extrinsic hormones, including peptide neurotransmitters that act like hormones. The intrinsic signals reside in the autonomic neuronal plexus within the gallbladder wall. Examples include acetylcholine and gastrin-releasing peptides which can induce contraction, while VIP, nitric oxide, and substance P lead to relaxation (Jazrawi 2000). Among the extrinsic hormones, CCK is considered the primary hormonal stimulus for gallbladder emptying (Otsuki 2000), and FGF15/19 was more recently discovered to be important in the relaxation of gallbladder smooth muscles (Choi, Moschetta *et al.* 2006). Others hormones such as somatostatin , motilin, secretin, histamine, prostaglandins, gastrin and glucagon have all been shown to have varying effects on gallbladder motility (Jazrawi 2000).

(1).CCK

CCK is a peptide hormone that was first extracted from intestinal mucosa (Ivy and Oldberg 1928). CCK influences gallbladder motility primarily at postprandial

periods. Fat in a normal meal induces CCK secretion from the duodenal I-cells (Buchan, Polak *et al.* 1978) and leads to gallbladder contraction within minutes (Shaffer, McOrmond *et al.* 1980). CCK also activates gallbladder contraction in a dose-dependent manner *in vitro* (Yau, Makhlouf *et al.* 1973).

CCK circulates in plasma at concentrations ranging from below 2 (fasting) to 10 pmol/l (postprandial) (Schjoldager 1994). It functions by interacting with the CCK receptors in the gallbladder smooth muscle cells (Steigerwalt, Goldfine *et al.* 1984; Liddle, Gertz *et al.* 1990; Mawe 1991; Schjoldager 1994) and by relaxing the sphincter of Oddi (SO) through VIP (Wiley, O'Dorisio *et al.* 1988; Pauletzki, Sharkey *et al.* 1993) or nitric oxide (Pauletzki, Sharkey *et al.* 1993). There is also evidence supporting the presence of CCK on presynaptic excitatory intrinsic neurons, which promote the release of acetylcholine and thus indirectly act on the gallbladder ganglion (Mawe 1991). However, cholinergic or adrenergic blockade does not affect the contractile effect by CCK (Hedner 1970; Amer 1972). The gallbladder resting tone can also be CCK-dependent because administration of the CCK antagonists L-364,718 or loxiglumide (a CCK-A antagonist) results in relaxation of the gallbladder (Liddle, Gertz *et al.* 1989; Schmidt, Creutzfeldt *et al.* 1991).

(2). FGF15/19

The effect of CCK on gallbladder motility is opposed by FGF 15/19. As discussed earlier, the mouse FGF15, or its human ortholog FGF19, is a secreted peptide hormone that is highly expressed in the ileum. In addition to its effects on liver bile acid homeostasis, FGF15/19 has recently been shown to regulate gallbladder filling directly

(Choi, Moschetta *et al.* 2006). Choi and colleagues report that gallbladders in mice lacking *Fgf15* are almost empty, even though the CCK level remains unchanged in these animals. Co-administration of FGF15 with CCK blocks the effect of CCK and leads to gallbladder filling within 15 minutes. In addition, there is no FGF15 expressed in the gallbladder, bile duct, or SO, and this observed gallbladder relaxation effect is considered another important endocrine function of FGF15/19 (Houten 2006). Because FGF19 is able to induce gallbladder filling in FGFR4$^{-/-}$ mice, the exact site of function and the downstream signaling mechanism is still unclear, although increased intracellular cAMP concentration correlates with the FGF15/19 treatment (Choi, Moschetta *et al.* 2006).

Role of the Gallbladder in the Enterohepatic Circulation of Bile Acids

The gallbladder and intestine react to the stimulus of eating by contracting and discharging bile into the duodenum, thus acting as a motor to drive the enterohepatic circulation of bile acids (Low-Beer, Heaton *et al.* 1971). Control of gallbladder motor functions involves a constant interplay between a large number of stimulatory and inhibitory hormones and neurotransmitters. It is now known that this model is oversimplified. Rather, the gallbladder response to stimuli is more complex, involving rapid alternation of emptying and refilling during the postprandial period (Jazrawi 2000). Notably, impairments in gallbladder motility are associated with the development of gallstone disease (Portincasa, Di Ciaula *et al.* 2008).

The distribution of the bile acid pool in hepatointestinal tissues and in the plasma changes during the day. It has been reported since the 1970s that postprandial bile acid

concentration is higher than the fasting level in both portal and peripheral blood (Lindblad, Lundholm *et al.* 1977; Roda, Aldini *et al.* 1978; Pennington, Ross *et al.* 1982). These findings suggest a role for the gallbladder and intestinal motility in the diurnal variation of the bile acid pool distribution. A negative correlation between gallbladder emptying rate and the size of the bile acid pool in humans has also been demonstrated by studies manipulating gallbladder emptying rate and monitoring bile acid pool size (Duane and Hanson 1978). A prolonged increase in gallbladder emptying induced by CCK octapeptide injections has been shown to also cause a reduction in the size of the bile acid pool due to an increase in fractional turnover rate, but there was no change in the synthesis rate of the two primary bile acids (Jazrawi and Northfield 1986).

Another component in the regulation of biliary motility is the Sphincter of Oddi (SO). It is a smooth muscle valve at the exit of the common bile duct into duodenum. Unlike the gallbladder, the SO relaxes in response to CCK stimulation (Toouli, Hogan *et al.* 1982), and it appears to be serve as a resistor to the flow of bile and pancreatic juice, as measurements in human show most flow occurs in phasic spurs (Torsoli, Corazziari *et al.* 1986; Worthley, Baker *et al.* 1989). Other than responding to CCK, the SO is also regulated by various other neurohormonal signals (Woods and Saccone 2007), and dysfunctions of the SO are associated with biliary pain or recurrent pancreatitis (Toouli and Craig 2000; Tanaka 2010).

CHAPTER II: TGR5 REGULATES GALLBLADDER MOTILITY

MATERIALS AND METHODS

Animal Procedures

TGR5$^{-/-}$ mice were provided by Galya Vassileva, Eric Gustafson and their colleagues at Schering-Plough (Vassileva, Golovko *et al.* 2006). All animal experiments were approved by the Institutional Animal Care and Use Advisory Committee of the University of Texas Southwestern Medical Center. Animals were fed global irradiated rodent chow (TD2916 Halan Teklad, Madison, WI) and water *ad libitum*. Both male and female mice have been tested. For measurement of bile acid pool size and composition, mice were fasted 4 hours before euthanasia with halothane. For *in vivo* gallbladder relaxation assays, mice were fasted for 14-18 hours, then treated with TGR5 agonists or vehicle control by intraperitoneal injections or gavage, and gallbladders were removed to measure bile volume within 30 minutes after the treatment. Tail-vein injection experiments were conducted as described by Choi *et al* (2006). FGF-19 was provided by Kelly Suino-Powell and Eric Xu.

Liquid Chromatography/Mass Spectrometry Analysis of Bile Acids

For measurement of bile acid pool size, bile acids were extracted and quantified as previously described (Lee, Schmidt *et al.* 2008). Briefly, the livers, gallbladders, and entire small intestines were collected, spiked with CDCA-D4, homogenized and extracted with 50 ml ethanol under reflux. Samples were filtered through No. 2 Whatman paper

before an aliquot of the extract was dried under a nitrogen stream and redissolved in methanol for liquid chromatography/mass spectrometry (Agilent Technologies, Palo Alto, CA) analysis. Bile acids were qualified by ionization in negative ion mode. Selective ion monitoring was used to detect the conjugated and unconjugated bile acids. Quantification was performed based on peak areas using external calibration curves of standards prepared in methanol. CDCA-D4 was used to calculate the recovery of bile acids after extraction relative to a blank control.

Bile Flow Measurement

Mice were fasted for 12 h before they were anesthetized by intraperitoneal injection of avertin. The abdomen was opened, the cystic bile duct ligated and a PE-10 catheter inserted at the distal end of the common bile duct prior to the branching point to the gallbladder and liver. The surgery was performed at 37°C. Hepatic bile was collected for 30 min. The volume of the bile was measured and normalized to elapsed time and the mouse body weight.

RT-qPCR Analysis

Total RNA was extracted from liver and intestine using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). After DNAse I (Roche) treatment, RNA was reverse transcribed into cDNA using the SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Primers for each gene were designed and validated as previously described (Bookout, Cummins *et al.* 2006). Primer sequences include *Tgr5*

forward 5'CCTGTCAGTCTTGGCCTATGAG3', reverse 5'GCCCAATGAGATGAGCGATA3'; *Cyp7a1* forward 5'AGCAACTAAACAACCTGCCAGTACTA3', reverse 5'GTCCGGA TATTCAAGGATGCA3'; *Cyp8b1* forward 5'GCCTTCAAGTATGATCGGTTCCT3', reverse 5'GATCTTCTTGCCCGACTTGTAGA3'; *18s* forward 5'ACCGCAGCTAGGAATAATGGA3', reverse 5'GCCTCAGTT CCGAAAACCA3'; *cyclophilin* forward 5'GGAGATGGCACAGGAGGAA3' 5'GCCCGTAGTGCTTCAGCTT3'. RT-qPCR reactions contained 25ng of cDNA, 5 ul of SYBR GreenER PCR Master Mix (Invitrogen) and 150 nM of each primer except for 18S (75 nM). The reactions were carried out in triplicate using an Applied Biosystems Prism 7900 HT instrument. Relative mRNA levels were calculated using either the comparative Ct or standard curve methods normalized to cyclophilin or 18S RNA, respectively.

Tensiometry Experiment

Gallbladders were removed from female mice anesthesized by halothane. Cross-sections of the gallbladder were cut. The right and left lateral aspects of the gallbladder were tethered with 0.2 mm pins and gallbladders were mounted in organ baths containing 8 ml of physiological saline solution (PSS, 120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 20.4 mM $NaHCO_3$, 1.6 mM $CaCl_2$, 10mM dextrose) at 37°C continuously bubbled with O_2:CO_2 (95 %: 5 %). Tension was measured with Grass FT03 force displacement transducers and a Powerlab 8SP digital recorder (AD Instruments, USA). Resting strip tension was adjusted to between 0.15 g and 0.2 g. Experiments were

started after a one-hour stabilization period. Tension responses to CCK-8 (1nM, Phoenix Pharmaceuticals, Inc) and TGR5 agonists or controls, were subsequently tested. LCA (50 μM), INT767 (10μM), INT747 (10μM) or DMSO control were tested as well as forskolin (2uM) as a positive control.

Gallbladder Explant Experiment and Determination of cAMP levels

Immediately after WT or *Tgr5*$^{-/-}$ mice were euthanized, gallbladders were removed, opened, and drained. The samples were incubated in ice cold PBS until all were ready. The tissues were then transferred into fresh ice-cold PBS and cut into three to four pieces. After all samples were ready, the gallbladder pieces were evenly distributed into different wells in a 6-well plate containing William's E medium prepared in 37 °C as described (Keitel, Reinehr *et al.* 2007). The tissue pieces were then incubated with either 25 μM INT-777 or vehicle control for 5 minutes, snap frozen in liquid nitrogen, and stored in -80°C. cAMP was extracted using 0.1N HCl as described (Choi, Moschetta *et al.* 2006). Intracellular cAMP levels were determined using a cAMP EIA kit (GE healthcare).

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). For comparison between 2 groups, the unpaired 2-tailed Student's t test was performed. One-way ANOVA followed by the Newman-Keuls procedure was used to compare more than 2

groups. All tests were performed using the software program GraphPad Prism 5. P values less than 0.05 were considered statistically significant.

RESULTS

TGR5 gene structure and QPCR primer design

TGR5 was originally thought to be a single exon gene (GenBank: BC119327.1). 5'-RACE and 3'-RACE experiments were conducted to analyze the gene structure of *TGR5*. We found that the gene has a non-coding 5'-exon preceding a short intron and the exon encoding the TGR5 protein (Figure 2-1).

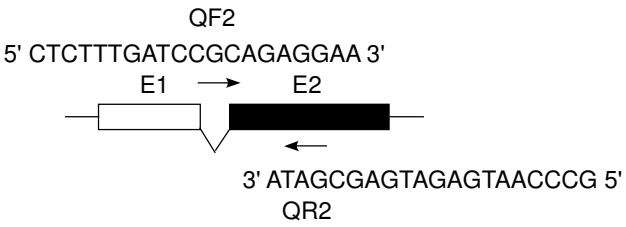


Figure 2-1. Exon structure of *Tgr5*
Exon structure of *Tgr5* was determined by 3'-RACE and 5'-RACE. The design of real time-PCR primers is shown. QF2 and QR2 indicate forward primer and reverse primer, respectively. QF2 crosses exon junction. E1 and E2 indicate the two exons.

TGR5 expression profile via RT-qPCR

Elucidation of the TGR5 gene structure made it possible to design RT-qPCR primers that span exon junctions such that we could effectively distinguish TGR5 cDNA from any contaminating genomic DNA in our samples (Figure 2-1). A comprehensive expression profile of TGR5 was then examined using tissues harvested from adult male C57BL/6 mice (Figure 2-2). The mRNA expression level of TGR5 is by far the most

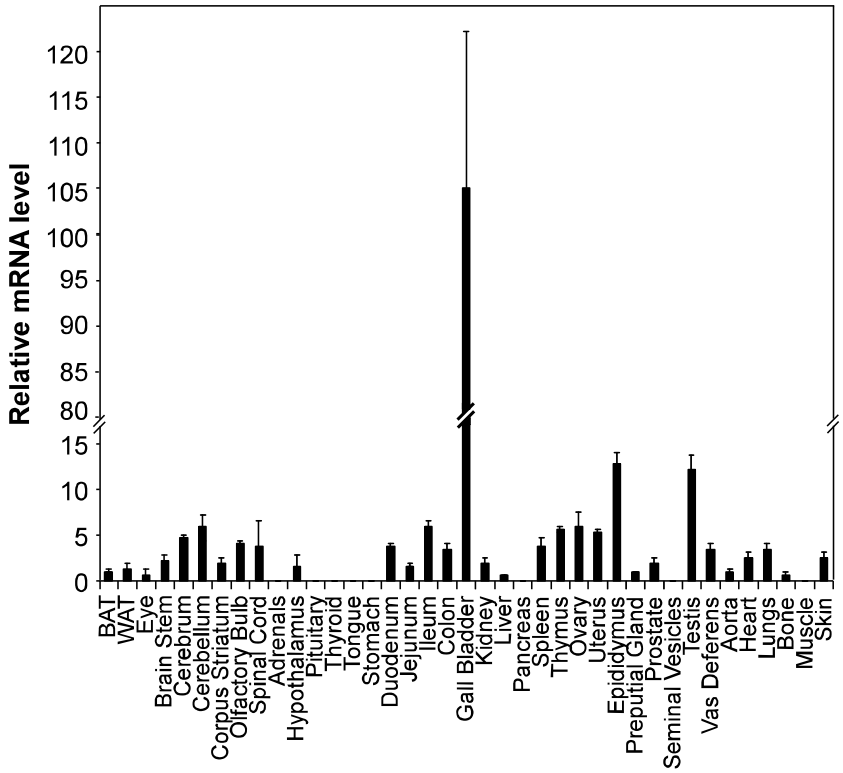


Figure 2-2. Tissue Distribution of TGR5 mRNA in pooled samples from C57/BL6 mice.
Samples were prepared as described in the method section. Relative mRNA levels were calculated using the standard curve methods and normalized to 18S RNA. The RT-qPCR experiment was performed by Zhu Wang.

abundant in the gallbladder, more than 20 times higher than tissues including testis, ovary, and ileum and more than 50 times higher than BAT (Figure 2-2). This is consistent with a previous report (Vassileva, Golovko *et al.* 2006).

TGR5 Localization in the Gallbladder

Immunostaining using anti-hTGR5 antibodies in human samples showed localization of TGR5 on the epithelium of the gallbladder (Figure 2-3). There was also spotty staining in the smooth muscle, suggesting possible localization in the neural plexus. However, attempts at immunohistochemistry and immunofluorescence using anti-mTGR5 antibodies on gallbladder tissues prepared from WT mice and *Tgr5*$^{-/-}$ mice revealed epithelial staining in both sets of samples (data not shown), suggesting

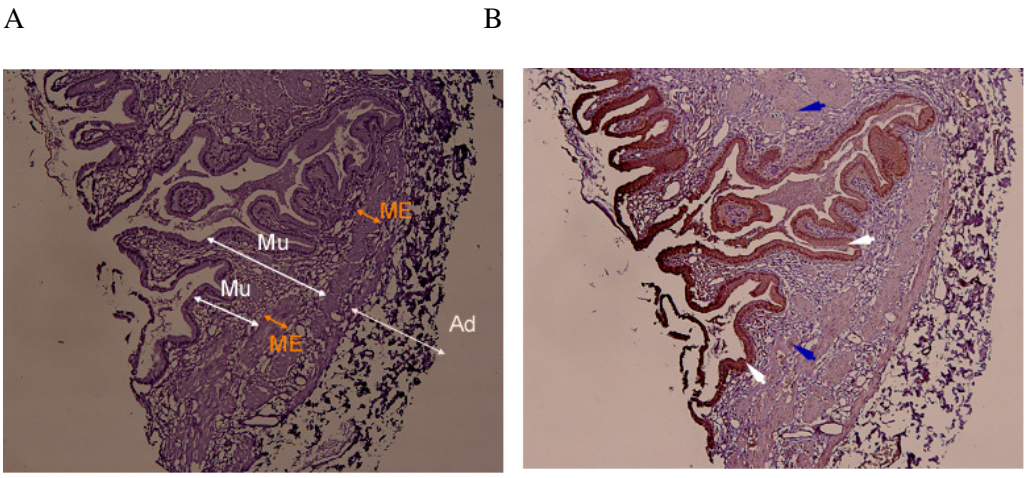


Figure 2-3. Immunohistochemistry of TGR5 in human gallbladder.
A, No primary antibody added; normal serum was used as control. The mucosa (Mu), muscularis externa (ME), and adventifia (Ad) of the gallbladder are labeled. **B,** TGR5 positive cells are stained. Arrows indicate staining in epithelial cells and spots in ME.

nonspecific staining. None of the published TGR5 immunostaining data (see introduction section for references) were presented with a knockout control, but *in situ* data in mice (Vassileva, Golovko *et al.* 2006) does suggest that TGR5 is epithelial localized. Keitel *et al.* (2009) also report epithelial specific staining (preincubation with anti-TGR5 antiserum abolishes the signal) in human gallbladders. Epithelial/neural localization seems consistent with TGR5 localization in cholangiocytes (Keitel, Ullmer *et al.* 2010) and enteric neurons. However, Lavoie *et al.*(2010) reported detection of both protein and mRNA in the smooth muscle of mouse gallbladder. It is possible that *Tgr5* is expressed in the neural plexus in gallbladder smooth muscle, resulting in the detection of *Tgr5* mRNA. It might be important to separate smooth muscle from neural plexus using laser capture microdissection prior to mRNA detection with RT-PCR. Good immunostaining data with knockout controls will be helpful as well.

TGR5 Stimulates Gallbladder Filling

While measuring the bile acid pool size, a trend toward reduced gallbladder volume in *Tgr5*$^{-/-}$ mice was noted (difference is significant when n>10/group is used). This trend was markedly enhanced by administration of 0.2% CA in the diet for 12 days (Fig. 2-4A). Under these conditions, gallbladder volume increased ~3-fold in WT mice but did not significantly change in *Tgr5*$^{-/-}$ mice. To assess whether acute TGR5 activation increases gallbladder volume, mice were injected with either the naturally-occurring TGR5 agonist, LCA, or the selective TGR5 agonist, INT-777 (a bile acid derivative, 6α-ethyl-23(S)-methylcholic acid, S-EMCA) (Pellicciari, Gioiello *et al.* 2009). Gallbladder

volume was measured 30 min after injection. Both agonists significantly increased gallbladder volume in WT but not *Tgr5*$^{-/-}$ mice (Fig. 2-4B and C). Thus, we conclude that TGR5 activation rapidly stimulates gallbladder filling.

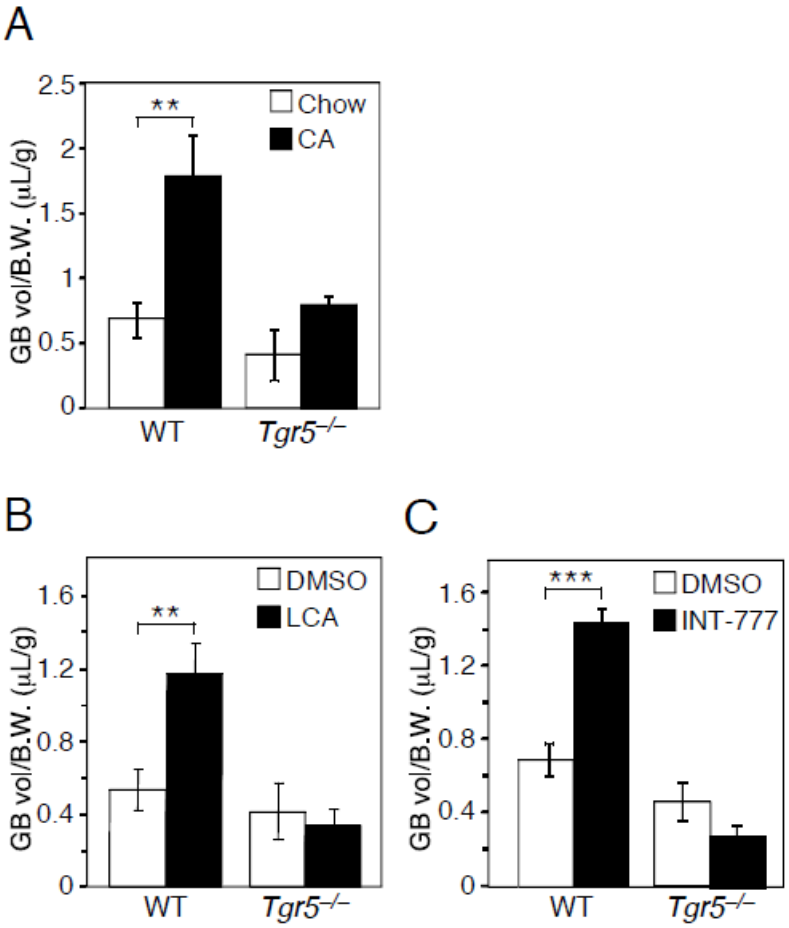


Figure 2-4. Effects of TGR5 agonists on gallbladder volume.

A, Gallbladder bile volumes were measured in WT and *Tgr5*$^{-/-}$ mice treated for 2 weeks with 0.2% CA in the diet. Control group were fed chow diet (n = 4-5/group). **B-C,** Gallbladder bile volumes were measured 30 min after intraperitoneal injection of (B) LCA (60 mg/kg), (C) INT-777 (60 mg/kg), or DMSO (n = 5-6/group). Volume data were normalized to body weight (B.W.). Male and female mice have remarkably similar responses. All data are the mean ± SEM. **P<0.01, ***P<0.001.

We also tested two other bile acid derivatives, INT-747 and INT-767, in the in vivo assay (Figure 2-5). INT-747, 6-ethyl-CDCA, is a semisynthetic derivative of CDCA and a selective FXR agonist; INT-767, the 23-sulfate derivative of INT-747, is the dual FXR/TGR5 agonist, and its potency as a FXR or TGR5 agonist is similar to that of INT-747 and INT-777, respectively (Rizzo, Passeri *et al.* 2010). We found that WT mice injected with INT-767 had enlarged gallbladders. In comparison, INT-747 did not change gallbladder volumes (Figure 2-5A). In addition, the INT-767 induced gallbladder filling

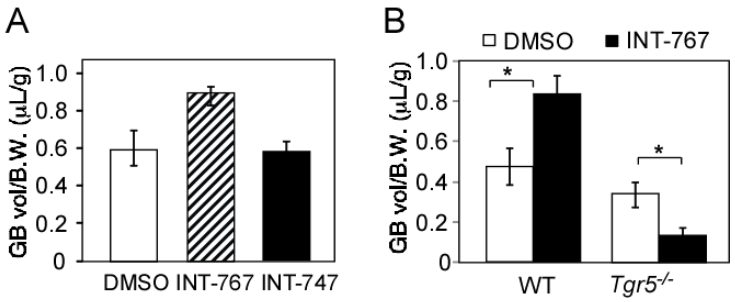


Figure 2-5. Effects of INT-767 (TGR5/FXR dual agonist) and INT-747 (FXR agonist) on gallbladder volume.

A, Gallbladder bile volumes in WT mice treated with INT-767, INT-747, or DMSO were compared. **B,** Effects of INT-767 on gallbladder bile volumes were tested in *Tgr5-/-* mice. Gallbladder bile volumes were measured 30 min after intraperitoneal injections of DMSO solutions of each compound as indicated. Data are the mean ± SEM. n=4-6/group. n.s. not significant. *P < 0.05.

was dependent on TGR5, as the dual compound reduced gallbladder volumes in *Tgr5*$^{-/-}$ mice (Figure 2-5C). INT-777 injection in *Tgr5*$^{-/-}$ also lead to reduced gallbladder volume, albeit to a lesser degree (Figure 2-4).

Possible explanations for gallbladder filling include: increased bile flow, directly relaxing gallbladder smooth muscle, indirectly modulating factor(s) that regulate gallbladder motility, or modulating Sphincter of Oddi (SO) activity. (We were not able to test SO because of the microscopic scale of this organ in mice. Reported functional studies are mostly performed in human patients by monitoring bile flow.) To result in rapid gallbladder filling under fasting conditions, when the phasic relaxation of SO is already low, one would need to look for further reduction in that phasic relaxation or reduction of bile flow from the common bile duct into duodenum. (See Background for more details.)

TGR5 Regulates Bile Flow

Bile flow measurements comparing WT and *Tgr5*$^{-/-}$ mice were conducted (Figure 2-6A). *Tgr5*$^{-/-}$ mice have significantly lower bile flow rate than WT mice, suggesting that TGR5 regulates bile secretion. INT-777 significantly increased bile flow (Fig. 2-6B). This result is consistent with the reduction in bile flow seen in *Tgr5*$^{-/-}$ mice. Given that TGR5 is expressed in cholangiocytes (Keitel, Donner *et al.* 2008; Keitel, Ullmer *et al.* 2010), these results raise the possibility that TGR5 directly regulates bile flow. LCA, on the other hand, decreased bile flow (Fig. 2-6D). This is not surprising because LCA has been long known to cause cholestasis (King and Schoenfield 1972). Because LCA decreases bile flow under conditions in which it increases gallbladder volume, the TGR5-dependent gallbladder filling effects of LCA cannot be accounted for by contributions from changes in bile flow. Taken together, the LCA and INT-777 data indicate that

TGR5 activation induces bile flow, but also that changes in bile flow are not required for TGR5-induced gallbladder filling.

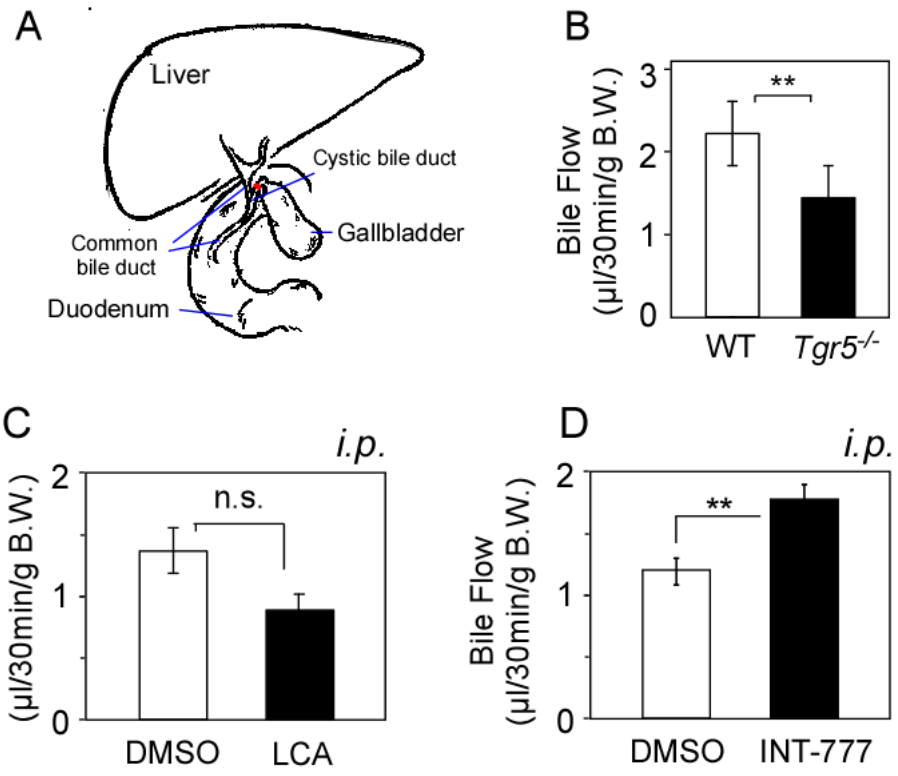


Figure 2-6. Effects of *Tgr5* deficiency or agonism on bile flow rate.

A, Surgeries on mice were performed to measure bile flow rate. The cystic bile duct was tied off (location indicated by red dot) to eliminate effects of the gallbladder on bile flow rate. A catheter was inserted at the distal end of the common bile duct prior to the branching point to the gallbladder and liver. Bile was then collected and flow rate calculated. **B,** Basal bile flow rates were measured in WT and *Tgr5-/-* mice (n= 6-8/group). **C, D,** Bile flow rates were measured in WT mice 15 min after intraperitoneal injection of (C) INT-777 (60 mg/kg), (D) LCA (60 mg/kg), or DMSO control (n = 4-6/group). Bile flow rates were normalized to body weight (B.W.). Male mice were used. All data are the mean ± SEM. **P<0.01; n.s., not significant.

TGR5 Relaxes Gallbladder Smooth Muscle Directly

We then looked at the gallbladder for possible explanations for the TGR5-induced gallbladder filling. To test whether TGR5 has direct effects on gallbladder smooth muscle, *ex vivo* tensiometry experiments were performed with gallbladders isolated from WT and *Tgr5*$^{-/-}$ mice. Both LCA and INT-777 markedly relaxed gallbladders from WT but not *Tgr5*$^{-/-}$ mice, and the relaxation was induced immediately upon addition of either compound (Fig. 2-7A and B). In contrast, forskolin, which induces cAMP levels, relaxed both WT and *Tgr5*$^{-/-}$ gallbladders (Fig. 2-7A and B).

Gallbladder tissues were examined for possible mechanisms, and a significant increase in cAMP concentrations was observed within 5 minutes of INT-777 incubation in gallbladders from WT but not *Tgr5*$^{-/-}$ mice (Figure 2-7C). We conclude that TGR5 acts directly on the gallbladder to cause smooth muscle relaxation via induction of cAMP levels.

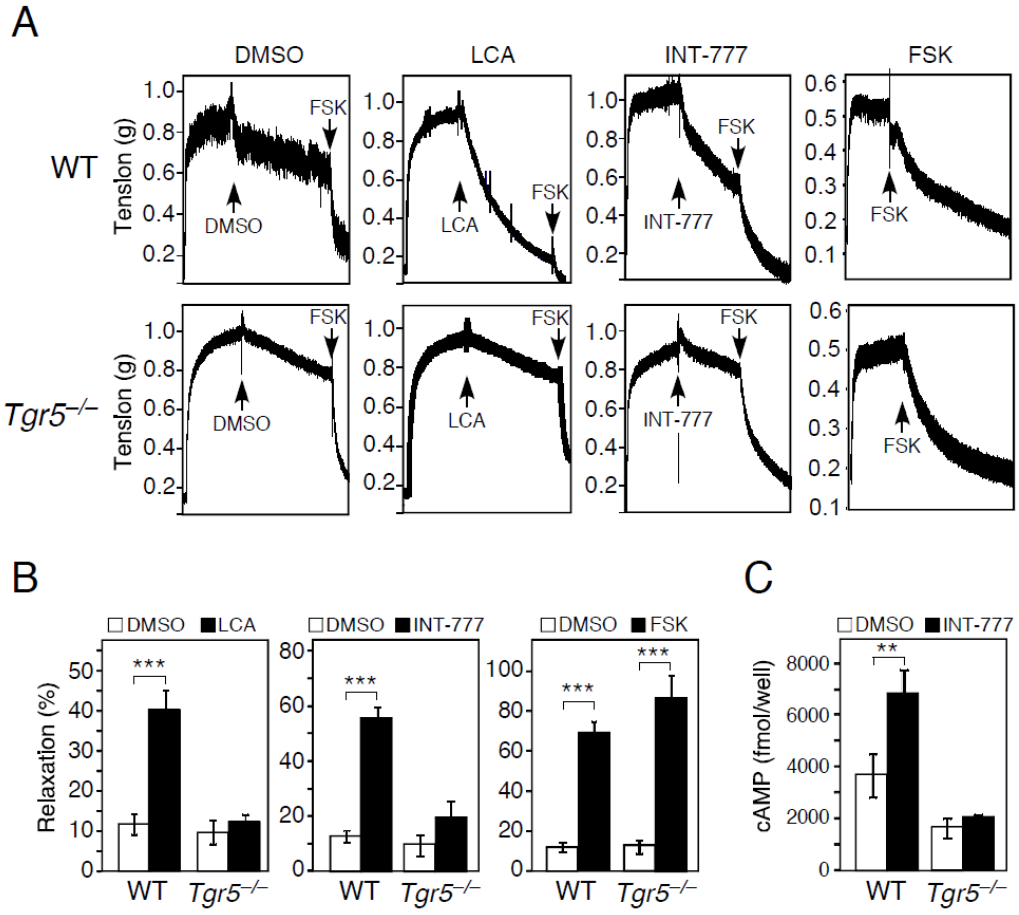


Figure 2-7. Effects of TGR5 activation on gallbladder smooth muscle *ex vivo*.

A, *Ex vivo* tensiometry was performed using gallbladder tissue from wild-type (WT, upper panels) or *Tgr5-/-* mice (lower panels). Gallbladders were pre-contracted with 1 nM CCK for 20 min, washed, and subsequently treated with either vehicle (DMSO), LCA (10 μM), INT-777 (10 μM) or forskolin (2 nM) for 20 min. DMSO, LCA and INT-777 treatments were followed by a second wash and treatment with forskolin (2 nM) for 20 min to ensure integrity of the tissue. Representative tension tracings are shown with treatment times indicated by arrows. **B,** Quantification of gallbladder relaxation was performed using tissue derived from WT or *Tgr5-/-* mice treated with vehicle (DMSO), LCA, INT-777 or forskolin (FSK) as in A. Data are the mean ± SEM of experiments performed in quadruplicate and are expressed as percent relaxation of CCK-induced contraction. ***P<0.001. **C,** Intracellular cAMP concentrations were measured in gallbladder tissue from WT or *Tgr5-/-* mice treated ex vivo with vehicle (DMSO; open bars) or INT-777 (25 μM; closed bars) for 15 minutes. Data are the mean ± SEM of assays performed 6-7 times. **P < 0.01. ***P<0.001.

TGR5 Regulation of Gallbladder Motility is CCK and FGF15-Independent

To test whether the TGR5 induced gallbladder filling is an indirect result of changes in CCK levels, we reserved the plasma from mice challenged with INT-777 or DMSO in the *in vivo* gallbladder relaxation assays, and measured CCK levels in these samples (Figure 2-8A). Neither *Tgr5* deficiency nor TGR5 activation by INT-777 changed CCK levels. In *ex vivo* tensiometry testing, CCK induced strong contraction in gallbladder strips prepared from both WT and $Tgr5^{-/-}$ mice (Figure 2-8B), suggesting the downstream signaling of CCK was also not affected either. In addition, when co-injected with CCK, INT-777 treatment completely reversed CCK-induced contraction of the gallbladder and stimulated gallbladder filling to levels similar to that observed in the absence of CCK (Figure 2-8C). This result suggests that TGR5-activated gallbladder filling is independent of CCK pathways.

We also tested the crosstalk between TGR5 and FGF15 pathways on gallbladder contractility. Because of the lack of good antibodies, we could not measure plasma levels of FGF15, but INT-777 administration induced gallbladder relaxation in $Fgf15^{-/-}$ mice (Figure 2-8D). This result suggests to us that TGR5 regulation of gallbladder motility is not FGF15-dependent.

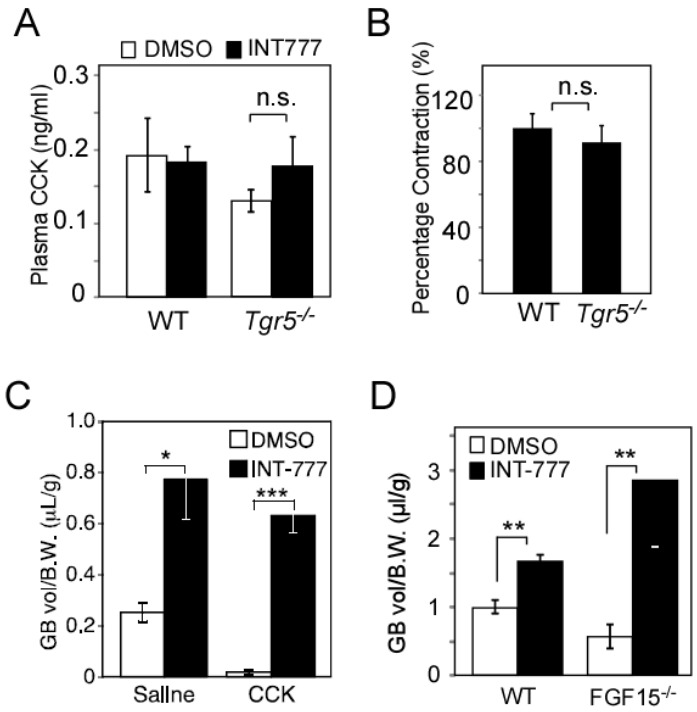


Figure 2-8. TGR5 action is CCK and FGF15-independent.

A, Plasma CCK levels were measured in WT and *Tgr5-/-* mice injected with DMSO or INT-777 (60mg/kg). Injection experiment performed as described. n=4-6/group. **B,** Quantification of gallbladder contracting tension using tissue derived from WT or *Tgr5-/-* mice treated with CCK (1nM) *ex vivo*. Contraction data is normalized to WT control and expressed as percentage contraction. n=4/group. Data are the mean ± SEM. n.s., not significant. **C,** Gallbladder volume was measured 30 minutes after intraperitoneal injection with CCK (200ng/kg) or saline control and INT-777 (60mg/kg) or DMSO control (n=5/group). This experiment was conducted by Serkan Kir. Gallbladder volumes were normalized to body weight (B.W.). **D.,** Gallbladder volume was measured 30 min after intraperitoneal injection of INT-777 (60 mg/kg) or vehicle (DMSO) into WT or *Tgr5*$^{-/-}$ mice (n = 4-6/group). Gallbladder volumes were normalized to body weight. All data are the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

DISCUSSION

TGR5 Regulates Gallbladder Motility

The gallbladder expresses *Tgr5* at the highest mRNA level of all tissues tested in mice (Our data; Vassileva, Golovko *et al.* 2006). Our findings support the hypothesis that TGR5 stimulates smooth muscle relaxation. Supporting evidence includes the following: (1) TGR5 is highly expressed in the gallbladder epithelium; (2) *Tgr5*$^{-/-}$ mice have reduced gallbladder volumes compared to WT mice; (3) administration of either the endogenous ligand, LCA, or the specific ligand of TGR5, INT-777, resulted in gallbladder filling *in vivo*, and this effect is lost in the *Tgr5*$^{-/-}$ mice; (4) natural ligands and synthetic agonists led to relaxation in WT gallbladder strips but not *Tgr5*$^{-/-}$ gallbladder strips, as measured by tensiometry *ex vivo*; (5) INT-777 treatment *ex vivo* led to increased cAMP concentration in gallbladder pieces from WT mice but not those from *Tgr5*$^{-/-}$ mice.

The ability of bile acids to attenuate gallbladder contractility has been reported previously (Rutishauser 1978). More recently, an independent report also making the connection with TGR5 was published from a group that studies the excitability of gallbladder smooth muscle (Lavoie, Balemba *et al.* 2010). The authors employed an *ex*

vivo assay measuring Ca^{2+} flashes in whole-mount gallbladder tissue as an indicator of spontaneous muscle activity. The authors report that: *Tgr5* mRNA and protein can be detected in the epithelium and muscle layer of the gallbladder; spontaneous activity in gallbladder smooth muscle was reduced by LCA treatment in gallbladder samples from WT mice but not *Tgr5*$^{-/-}$ controls; the ability of LCA to inhibit muscle activity is abolished by a PKA inhibitor. In addition, it was shown that the K_{ATP} channel blocker, glibenclamide, also reduces the spontaneous gallbladder smooth muscle activity. It is thus suggested that TGR5 regulates smooth muscle activity via cAMP-PKA- K_{ATP} pathway. These observations present strong *ex vivo* evidence complementary to our findings *in vivo*. I should probably note that many attempts to test several different inhibitors, including two PKA inhibitors, have failed in my tensiometry assays. Reasons for the failure are currently unclear.

We found that bile acids regulate gallbladder motor activity and inhibit CCK-induced contraction via TGR5. Because the gallbladder concentrates bile up to 10 fold by extracting water and electrolytes (see Background for details), the concentration of bile acids in the gallbladder would be directly correlated with the amount of time the gallbladder had to collect bile. Thus, the longer the gallbladder had to collect bile after feeding, the higher the bile acid concentration would likely be. Based on our findings, we suggest a two-pronged "distal/proximal" signaling mechanism for controlling gallbladder filling. FGF15/19, which is induced by bile acids in the ileum, represents a distal hormonal signal that effectively primes the gallbladder for refilling following a meal (Choi, Moschetta *et al.* 2006). The presence of TGR5 in gallbladder provides a

reinforcing proximal mechanism for detecting local bile acid concentrations and adjusting gallbladder volume accordingly.

The concentrations of bile acids in the gallbladder bile are in the milli-molar range (see Background), so how TGR5 activity might be effectively regulated in this environment considering its EC_{50}s (0.3-7 μM for various bile acids) is a question that remains.

It is difficult to demonstrate effective concentration of bile acids passing the protective mucous layer above the epithelium, but it is very unlikely that under normal physiological conditions, the epithelial cell membrane is in contact with bile acids at concentrations at or higher than millimolar range, for the membrane integrity would be affected under such conditions, even for epithelial cells (Jean-Louis, Akare *et al.* 2006).

TGR5 Stimulates Bile Secretion

Another hypothesis that is supported by this project is a role for TGR5 in regulating bile flow. We showed that *Tgr5*$^{-/-}$ mice have a decreased bile flow rate, and that a TGR5-specific ligand increases bile flow. In addition, Alphini (1997) reported that bile acids stimulate secretory capacity of isolated cholangiocytes. This suggests a role of TGR5 in the hepatic secretion of bile. It has been reported that TGR5 induces chloride secretion from the isolated gallbladder epithelium via CFTR (Keitel, Reinehr *et al.* 2007). Chloride secretion is integral to bile formation (Meier and Stieger 2000). Thus it is possible that bile acids regulate biliary secretion in a feedforward manner via TGR5. In

other words, TGR5 provides a means for biliary ducts to sense their "work load" and adjust accordingly.

Future Studies

It will be interesting to investigate the mechanism underlying the two proposed roles of TGR5. Further understanding will require a careful analysis of the expression of TGR5 in these different cell types with laser capture or other techniques. Studies to further define the relationship between TGR5 and FGF15/19 would be interesting.

The effect of TGR5 activation on SO remains unclear. Expression analysis together with technical expertise working with SO from mice could help determine the role of TGR5 (or the lack thereof) in this organ.

Further, the added role of TGR5 introduces a new set of tools to study the different functions of the hepatobiliary system, and new information on the function and regulation of these "leaky membranes" could provide important information on the epithelium in other organs.

CHAPTER III: TGR5 IN BILE ACID METABOLISM

MATERIALS AND METHODS

Animal Procedures

Age matched (3-4 months old) male mice had free access to water and were fed ad libitum (for 2 or 17 weeks) from one of these diets: powdered global irradiated rodent chow (TD2916 Halan Teklad, Madison, WI); this powdered diet supplemented with 0.2% CA; or global irradiated rodent chow (TD2916) in pellet form; western diet (TD88137, Harlan Teklad, Madison, WI) in pellet form, containing 21% (w/w) total lipid (42% calories as anhydrous milk fat [65% saturated, 32% monounsaturated, 3% polyunsaturated fats]) and 0.2% (w/w) total cholesterol (of which 0.05% is contributed by milk fat and 0.15% is added). Body weight was recorded weekly, and food intake was measured twice weekly. Total body mass was analyzed before and after the experiment by NMR using the Mini-spec mq spectrometer (EchoMRI-100). At the end of the feeding period, mice were anesthetized with halothane prior to tissue harvest. Blood was kept on ice in heparin-coated tubes (Microvette 500 LH; Sarstedt, Inc.) and centrifuged (3000x for 15 min at 4˚C), and the plasma was stored at -20˚C until analysis. Tissues were harvested, snap frozen in liquid nitrogen, and kept at -80˚C until analysis. Bile flow measurement was carried out in mice fed on global chow diet. Further details were described in the method section of chapter 2. Male mice were used unless specified. All experiments were approved by the Institutional Animal Care and Use Advisory Committee at the University of Texas Southwestern Medical Center.

Hepatic Cholesterol Measurement

Cholesterol was extracted from saponified liver (150mg) in chloroform:methanol (2:1, v/v). Extracts were then washed once in 50mM NaCl and twice in 0.36 M $CaCl_2$/methanol. The organic phase was separated and brought up to 5 ml with chloroform. Ten microliters of chloroform:Triton X-110 (1:1, v/v) was added to duplicate 100 µl aliquots of each extract and standards (Sigma Diagnostics), which were then air dried overnight. Colorimetric enzymatic assays were performed (Roche Diagnostics). After 15 min, optical densities of three aliquots of each sample were read at 510 nm and compared to prepared standards, as instructed.

Fecal Bile Acid Excretion

Feces were collected from individually housed mice over a continuous 72-h period. Bile acids were extracted, as previously described (Turley, Schwarz *et al.* 1998). Briefly, feces were dried in an 80°C oven, weighed, and ground into powder. [Carboxyl-^{14}C] cholate was added to 1g of stool as an internal control and samples were treated with 10 ml sodium borohydride. After alkaline hydrolysis at 100°C for 12 hours, samples were eluted with methanol using C18 Bond Elute columns (Varian). Bile acid content in extracts was quantified by 3α-dehydroxysteroid dehydrogenase (3HSD) assays. Daily excretion was expressed as µmol/day/100 g body weight.

Liquid Chromatography/Mass Spectrometry Analysis of Bile Acids

For measurement of bile acid pool size, bile acids were extracted and quantified as previously described (Lee, Schmidt *et al.* 2008). Briefly, the liver, gallbladder, and entire small intestine were collected, spiked with CDCA-D4, homogenized and extracted with ethanol under reflux. Samples were filtered through No. 2 Whatman paper before an aliquot of the extract was dried under a nitrogen stream and redissolved in methanol for liquid chromatography/mass spectrometry analysis. Bile acids were quantified by ionization in negative ion mode. Selective ion monitoring was used to detect the conjugated and unconjugated bile acids. Quantification was performed based on peak areas using external calibration curves of standards prepared in methanol. CDCA-D4 was used to calculate the recovery of bile acids after extraction relative to a blank control.

Statistical analyses

Values are expressed as mean ± standard error of the mean. Significant differences between mean values were evaluated using two-tailed, unpaired Student's t test when two groups were analyzed or one-way ANOVA followed by Student Newman-Keuls test for three or more groups. All tests were performed using the software program GraphPad Prism 5.

RESULTS

Effects of TGR5 on Bile Acid Metabolism

Bile acid parameters were analyzed in WT and *Tgr5*$^{-/-}$ mice fed either regular chow diet or chow supplemented with 0.2% CA, both in powdered form. Consistent with previous reports (Maruyama, Tanaka *et al.* 2006), bile acid pool size was significantly smaller in both male and female *Tgr5*$^{-/-}$ mice (male, 34.94 ± 1.59 µmol/100g B.W. vs. WT control 49.12 ± 3.34 µmol/100g B.W. $p<0.01$; female 52.30 ± 1.86 µmol/100g B.W. vs. WT control 83.78 ± 3.17 µmol/100g B.W. $p< 0.005$) (Figure 3-1A). Further, the decrease in bile acid pool size was accompanied by a decrease in the fraction of tauro-β-MCA in the pool (Figure 3-1B, C). Because MCA is more hydrophilic than CA, the increased CA/MCA in the bile acid pool would lead to increased hydrophobicity. This is especially puzzling, because increased hydrophobicity increases susceptibility to cholesterol gallstone disease (CGD) (Hay and Carey 1990), but *Tgr5*$^{-/-}$ mice have decreased susceptibility to CGD.

A detailed representation of the bile acid composition in the total bile acid pool can be found in Figure 3-2.

Bile acid flow rate was significantly slower in *Tgr5*$^{-/-}$ mice (1.44 ± 0.11 µl/min/g vs. WT control 2.2 ± 0.24 µl/30min/g, $p < 0.01$) (Figure 3-1D) consistent with the decrease in bile acid pool size. Fecal bile acid levels in chow-fed mice, which often correlates with new bile acid synthesis, were not significantly different (1.47 ± 1.09 vs. WT control 1.95 ± 0.53, p = n.s.) (Figure 3-1F).

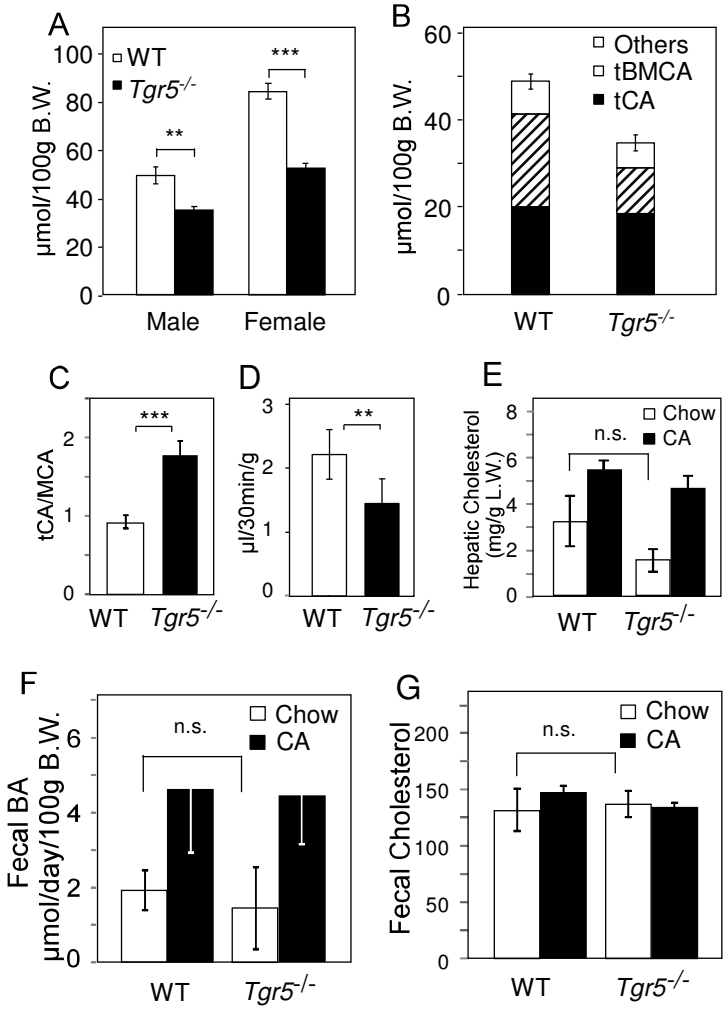
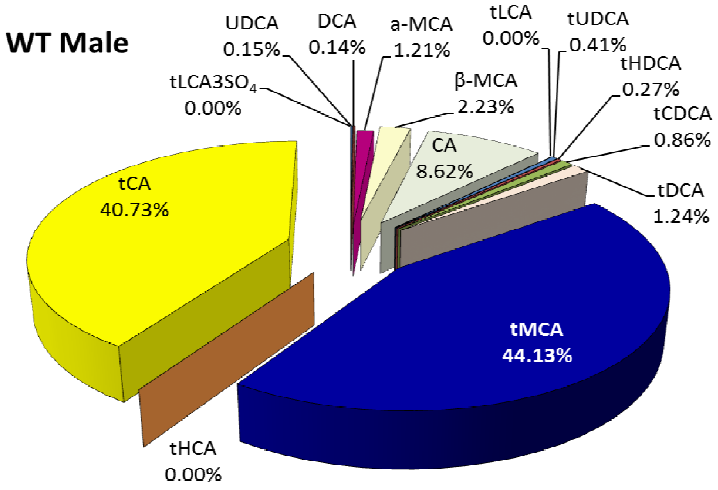
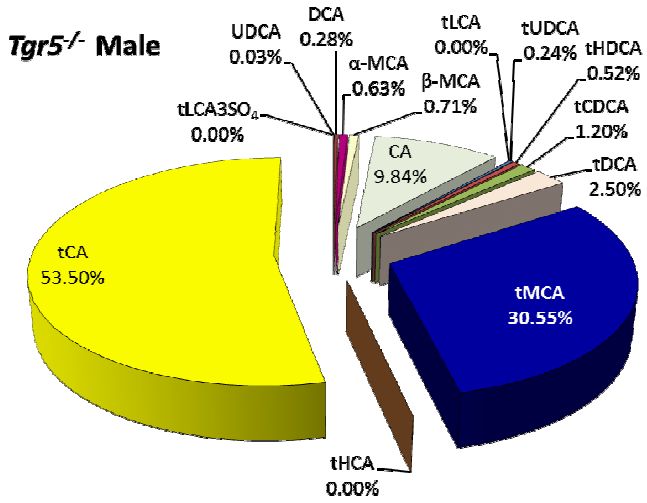


Figure 3-1. Bile parameters in *Tgr5*$^{-/-}$ mice.

A, Bile acid pool size was measured in male and female WT and *Tgr5*$^{-/-}$ mice (n = 6/group). **B** and **C,** Bile acid composition, including tauro-β-muricholic acid (tBMCA) and tauro-cholic acid (tCA), was measured by liquid chromatography/mass spectrometry in WT and *Tgr5*$^{-/-}$ mice (n = 6/group) fed chow diet. **D,** Bile flow rate was measured in WT and *Tgr5*$^{-/-}$ mice on chow diet (n = 6/group). **E,** Hepatic cholesterol levels from WT and *Tgr5*$^{-/-}$ mice on both chow and 0.2% CA diet was measured. P= 4-5/group. **F** and **G**, Fecal BA and cholesterol levels was measured in WT and *Tgr5*$^{-/-}$ mice on chow and 0.2% CA diet (p = 4 – 5/group). All data are the mean ± SEM. Error bars in B indicate SEM of the portion of the bile acid pool excluding CA and MCA. **P<0.01, ***P<0.001, n.s. not significant. B.W. body weight, L.W. liver weight.

Hepatic cholesterol level trended lower in the *Tgr5*$^{-/-}$ mice compared to the WT mice, but the difference was not significant (1.58 ± 1.09 mg/g L.W. vs. WT control 3.21 ± 5.43 mg/g L.W. p = n.s.) (Figure 3-1E). Fecal levels of bile acid and cholesterol excretion both remained unchanged (Figure 3-1F,G).





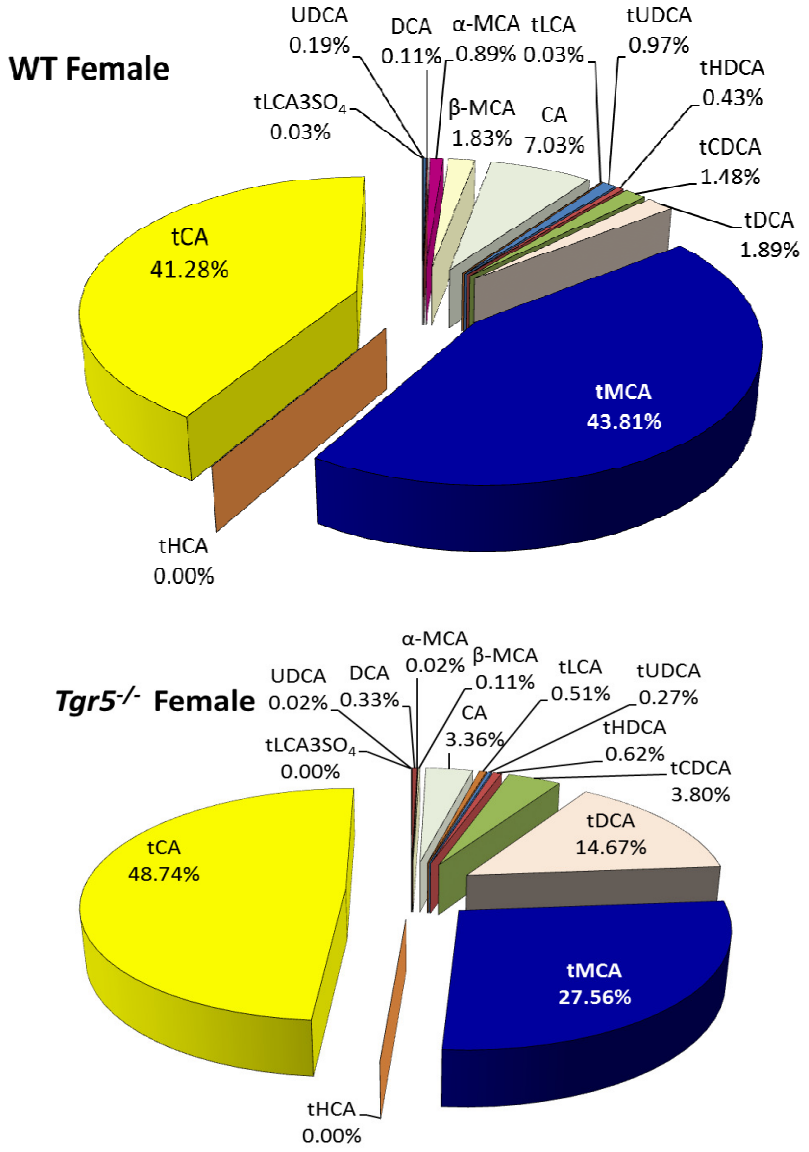


Figure 3-2. Effects of *Tgr5* deficiency on bile acid composition.

Total bile acids were extracted from the gallbladder, liver, intestine, and portal blood and quantitated by liquid chromatography/mass spectrometry. The results shown are bile acid pool size and composition in in 3 month old male and female mice (n = 6/group) from WT and *Tgr5*$^{-/-}$ mice as indicated. Taurocholate (tCA) and tauromuricholates (tMCA, includes α-, β, and ω-muricholate) compose the majority of bile acids in the mouse.

Expression of Hepatic Genes Involved in Bile Acid Metabolism Is Not Altered in *Tgr5*$^{-/-}$ Mice

To understand the mechanism underlying the change in bile acid pool size and

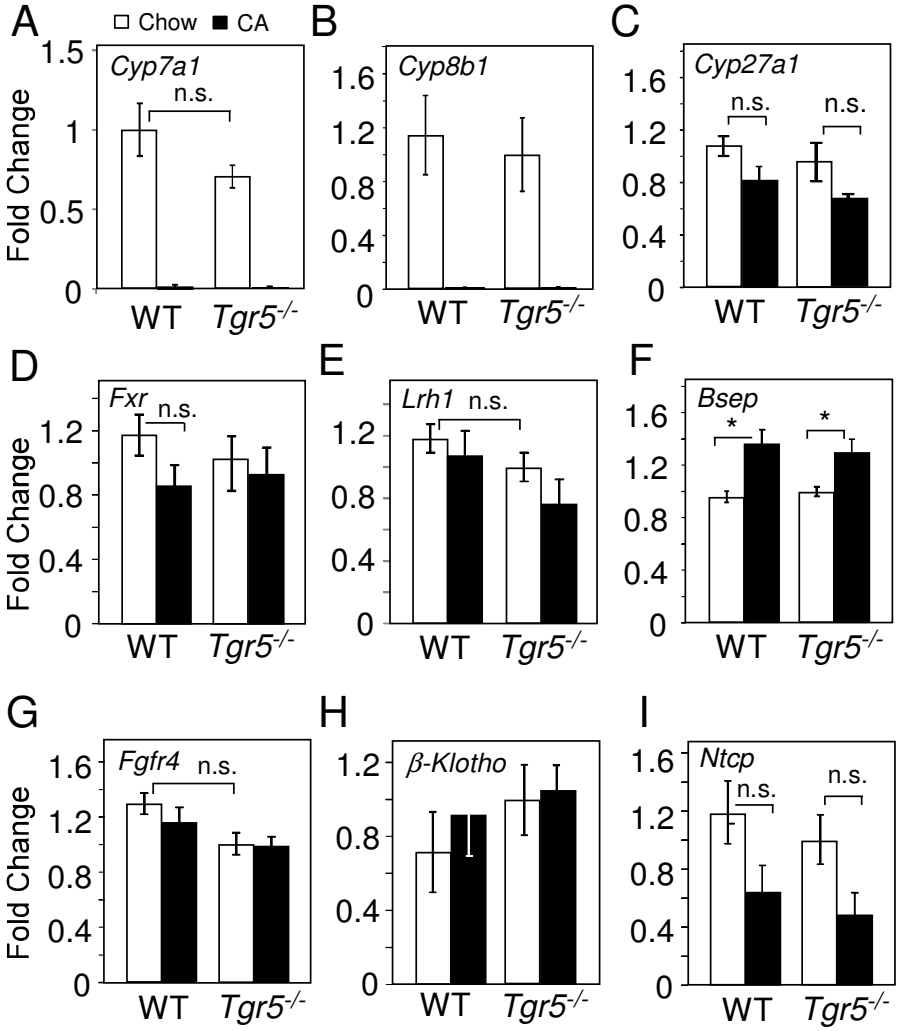


Figure 3-3. Effects of *Tgr5*$^{-/}$ deficiency on mRNA levels of hepatic genes regulating bile acid metabolism.

Tgr5-/- and WT control mice were fed with 0.2% CA diet for 12 days and mRNA prepared from liver. RT-qPCR was used to measure the mRNA levels of hepatic genes. Above shown are *Cyp7a1, Cyp8b1, Cyp27a1, Fxr, Lrh1, Bsep, Fgfr4, β-klotho* and *Ntcp*. Data are the mean ± SEM (n=3-5 per group). * $p<0.05$, n.s., not significant.

composition, we examined the effect of *Tgr5* deficiency on hepatic expression of key enzymes involved in bile acid biosynthesis. The expression of *Cyp8b1* is of particular interest, because it 12α-hydroxylates bile acid intermediates and has a dramatic effect on CA/MCA ratio (CA/CDCA in human) (Pandak, Bohdan *et al.* 2001).

However, the expression levels of key cytochrome p450 enzymes, including *Cyp7a1, Cyp8b1, and Cyp27a1*, are not significantly changed (Figure 3-3 A, B, C), perhaps not surprisingly, considering that TGR5 is expressed in cholangiocytes instead of hepatocytes (Keitel, Ullmer *et al.* 2010). In addition, CA feeding in the *Tgr5*$^{-/-}$ mice resulted in effective repression of *Cyp7a1* and *Cyp8b1* levels, suggesting that *Tgr5* is not involved in the feedback repression of bile acid biosynthesis (Figure 3-3).

The expression levels of other genes involved in bile acid biosynthesis and transport are not changed either. For example, mRNA level of *Ntcp,* the Na(+) taurocholate cotransporting polypeptide, a FXR/SHP target, is repressed by CA feeding in both WT and *Tgr5*$^{-/-}$ mice. *Bsep,* a FXR target gene, is induced upon CA feeding in both WT and *Tgr5*$^{-/-}$ mice as well. No significant change was detected in basal levels and CA regulation of *Fxr, Lrh1* or *Bsep* (Figure 3-3). *Fgfr4* levels trended higher and *β-Klotho* trended lower in *Tgr5*$^{-/-}$ mice.

Dysregulation of Ileal Transporters

So far, we observe in *Tgr5*$^{-/-}$ mice an increased CA/MCA ratio, but no change in hepatic expression of cytochrome p450 enzymes including *Cyp8b1*. There is no significant change in levels of hepatic cholesterol, fecal bile acid excretion, nor fecal cholesterol excretion in *Tgr5*$^{-/-}$ mice. In addition, we report increased hydrophobicity level in bile, and this is the opposite of what would be expected considering a previous report showing *Tgr5*$^{-/-}$ mice are resistant to CGD. This resistance was attributed to increased phospholipid content in bile in *Tgr5*$^{-/-}$ mice; biliary bile acid and cholesterol level were not different (Vassileva, Golovko *et al.* 2006).

One possible explanation for the change in bile acid composition is that the enterohepatic circulation, or more specifically, the intestinal absorption of bile acids is altered in *Tgr5*$^{-/-}$ mice. To address this we then examined ileal expression levels of genes involved in bile acid metabolism by RT-qPCR.

Fgf15 expression level was unchanged in the ileums of *Tgr5*$^{-/-}$ mice (Figure 3-4 A), and CA feeding induced *Fgf15* expression similarly in both WT and knockout animals. Because *Cyp7a1* repression by CA is intact in *Tgr5*$^{-/-}$ mice, these data indicate that TGR5, the bile acid receptor, is not required for *Fgf15* feedback repression of bile acid synthesis involved in bile acid feedback regulation, and support the hypothesis that TGR5, a bile acid receptor, is not involved in bile acid feedback regulation.

Bile acid transporters in the ileum do have an interesting expression profile. The basal expression of *Ostα* and *Ostβ* is lower in *Tgr5*$^{-/-}$ mice, a significant

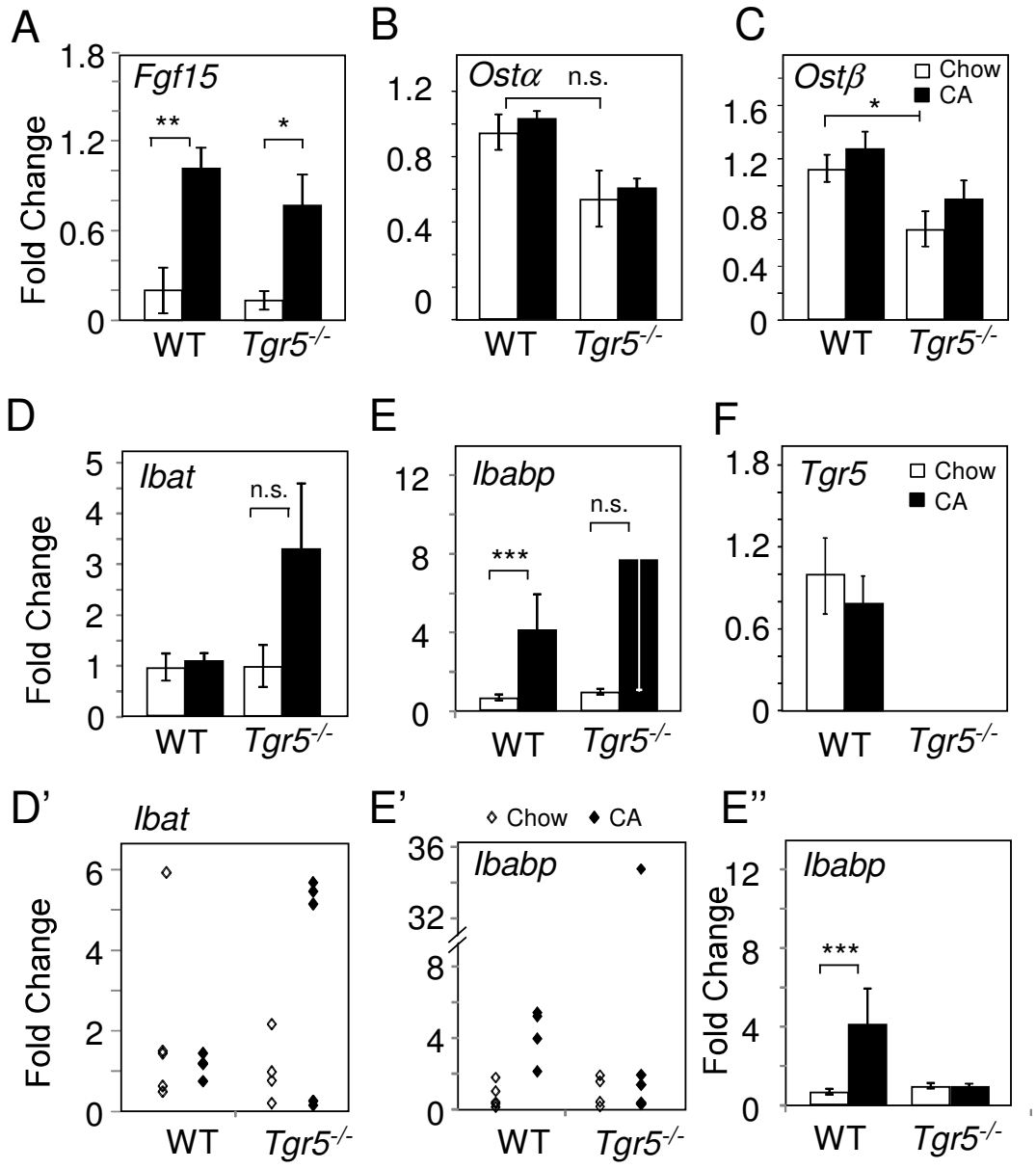


Figure 3-4. Effect of CA feeding on mRNA levels in the ileums of *Tgr5-/-* mice.

RNA was prepared from ileum of *Tgr5*$^{-/-}$ and WT mice fed on 0.2% CA or chow diet. RT-qPCR was used to measure the mRNA levels of *Fgf15, Ostα, Ostβ, Tgr5, Ibat*, and *I-babp*. The scatter plot of expression levels of *Ibat* and *Ibabp* in individual mice was shown. Data are the mean ± SEM. n=3-5/group. * p<0.05, **p<0.01, ***p<0.005, n.s., not significant.

difference, in the case of *Ostβ* but not *Ostα* (p=0.07) (Figure 3-4 B, C)). Decreased expression of these transporters could lead to decreased basolateral efflux of bile acids and subsequently reduced bile acid pool size.

Perhaps more interestingly, the expression of *Ibat and I-babp* was dysregulated in *Tgr5*$^{-/-}$ mice (Figure 3-4). In WT mice, *I-babp,* a FXR target, was significantly upregulated by CA feeding, and the difference was not significant in *Tgr5*$^{-/-}$ mice (Figure 3-4 E). Statistical analysis of *Ibat* level in both WT and *Tgr5*$^{-/-}$ mice suggest that CA feeding do not significantly change *Ibat* expression in either genotype, but its expression level seem to be highly variable in *Tgr5*$^{-/-}$ mice too (Figure 3-4 D).

DISCUSSION

It was previously shown that *Tgr5*$^{-/-}$ mice are resistant to lithogenic diet-induced gallstone formation, which was attributed to an increase in *Cyp7a1* expression and a corresponding reduction in the cholesterol saturation index of the bile (Vassileva, Golovko *et al.* 2006). Here we presented preliminary data on the changes in bile acid metabolism in *Tgr5*$^{-/-}$ mice. We confirmed previous reports of a decreased bile acid pool in the *Tgr5*$^{-/-}$ mice, and reported a lowered MCA level, an increased CA/MCA ratio in the bile acid pool, and a decreased hepatic bile flow in the *Tgr5*$^{-/-}$ mice. Expression levels of genes involved in bile acid biosynthesis in the liver are not altered. However, we present preliminary data showing dysregulated transport of bile acids and cholesterol in *Tgr5*$^{-/-}$ mice. The mRNA levels of ileal *Ostα* and *Ostβ* are lowered in *Tgr5*$^{-/-}$ mice, while *Ibat* and *I-babp* expression is dysregulated.

Together, we are presented with two questions: (1) what causes the significantly decreased bile acid pool size and increased CA/MCA in *Tgr5*$^{-/-}$ mice? Hepatic synthesis does not seem to be responsible for this change; (2) what causes the increased CGD resistance in *Tgr5*$^{-/-}$ mice other than increased biliary phospholipid level? Increased hydrophobicity of the bile acid pool resulted from the increased CA/MCA ratio in *Tgr5*$^{-/-}$ mice would ordinarily lead to increased CGD. We are currently investigating the mechanism(s) underlying these two apparent contradicting phenotypes found in *Tgr5*$^{-/-}$ mice.

Altered Bile Acid pool size and composition

As discussed in the first chapter, only 5% of the bile acids pool is newly synthesized everybody, with the same amount excreted through fecal excretion. 95% of the bile acid pool is reabsorbed in the intestine and recirculated in the enterohepatic system repeatedly. An altered enterohepatic circulation can thus effectively change the bile acid pool. We then looked the dysregulated bile acid transporters more closely.

Dysregulated Bile Acid Transporters

A closer examination revealed that the expression of *Ibabp* in three out of four *Tgr5*$^{-/-}$ mice is not upregulated by CA feeding (Figure 3-4 E'), and this failure to respond to the increased CA in the intestine in comparison to the WT response can be seen clearly when the one outlier sample is deleted ($p < 0.05$, Figure 3-4 E''). As shown in the same figure, CA feeding in WT mice significantly induced *Ibabp* expression ($p<0.005$) as expected (Grober, Zaghini *et al.* 1999; Hwang, Urizar *et al.* 2002; Landrier, Grober *et al.* 2002).

The expression of *Ibat* is also largely dysregulated in *Tgr5*$^{-/-}$ mice, although no clear pattern is seen (Figure 3-4 D'). Because Ibat expression is not affected by Ibabp and IBAT is functionally upstream of IBABP (Nakahara, Furuya *et al.* 2005) and the normal function of IBAT is essential for the absorption of conjugated bile acids, which in turn activates *Ibabp* expression, it is likely that the dysregulated IBAT is upstream of the IBABP phenotype in *Tgr5*$^{-/-}$.

It has been shown that cAMP can induce a rapid increase of hepatic Na+ dependent bile acid transport by inducing vesicular trafficking of NTCP from the intracellular pool to the basolateral membrane (Mukhopadhayay, Ananthanarayanan *et al.* 1997; Dranoff, McClure *et al.* 1999; Alrefai and Gill 2007). Apart from its effects on sinusoidal membrane, cAMP has also been shown to increase Mrp2 (Roelofsen, Soroka *et al.* 1998), Mrd2 and Mrd3 (Gatmaitan, Nies *et al.* 1997)and BSEP (Kipp, Pichetshote *et al.* 2001) in canalicular membranes. The effect of cAMP and the molecular mechanism has been extensively reviewed (Anwer 2004; Alrefai and Gill 2007), while the physiological significance is still not well understood. The phenotype of *Tgr5*$^{-/-}$ in hepatic bile flow (Figure 2-6) and ileal bile acid transporters (Figure 3-4) led us to suggest the possibility that TGR5 respond to bile acids to induce cAMP and regulate bile acid transporters in the liver and in the ileum. Decreased TGR5 activity in hepatic bile acids transporters and in the gallbladder in response to the decreased serum bile acid level could underlie the immediate decrease in gallbladder volume upon cholestyramin ingestion (van Ooteghem, Moschetta *et al.* 2002). Failure to increase IBAT activity can also explain the decreased bile acid pool size and the increased CA/MCA ratio in *Tgr5*$^{-/-}$ mice (Figure 3-1), which is also observed in *Ibat*$^{-/-}$ mice (Dawson, Haywood *et al.* 2003). Further, the involvement of CFTR in TGR5 activity (Keitel, Cupisti *et al.* 2009) and bile acid transport (Cohn, Strong *et al.* 1993; Stelzner, Somasundaram *et al.* 2001; Bijvelds, Jorna *et al.* 2005) is intriguing.

To test this hypothesis, many physiological and mechanistic experiments can be done as shown by the above mentioned reports. A direct test of the ileal bile acid

transport in *Tgr5*$^{-/-}$ is to measure the mucosal absorption of radiolabeled cholic acid or muricholic acid *ex vivo* (Stelzner, Somasundaram *et al.* 2001; Bijvelds, Jorna *et al.* 2005) using ileum segments isolated from WT and *Tgr5*$^{-/-}$ mice.

Resistance to Cholsterol Gallstone Disease (CGD)

Another phenotype of *Tgr5*$^{-/-}$ mice that is not well-understood is the resistance to CGD. Vassileva *et al.* (2006) reported that in WT mice with normal expression of *Tgr5* gene, 54% developed cholelithiasis with gallstones when fed on lithogenic diet, while *Tgr5*$^{-/-}$ mice did not show a single occurrence of gallstone. This resistance to CGD observed in *Tgr5*$^{-/-}$ mice is the opposite of what is expected with the dramatically increased hydrophobicity in the bile acid pool resulted from the increased CA/MCA ratio ($p < 0.001$) in these knockout animals. Vassileva *et al.* (2006) reported unchanged cholesterol and bile acid concentration in the gallbladder bile, but increased phospholipid concentration in *Tgr5*$^{-/-}$ mice (43.5±7.5 μmol/ml vs. WT 32.0±9.3 μmol/ml $p = 0.03$), which can contribute to the resistance to gallstone disease.

As discussed earlier, an important factor in the pathogenesis of CGD is the presence of nucleating proteins, namely, mucin proteins. Because the activities of different bile acids to stimulate mucin secretion (Klinkspoor, Kuver *et al.* 1995) correlate well with their potencies as TGR5 ligands (Kawamata, Fujii *et al.* 2003), and the molecular mechanism has not been understood, we then conducted a preliminary experiment looking at the mucin secretion in these animals.

Mucin Production in Tgr5$^{-/-}$ Mice

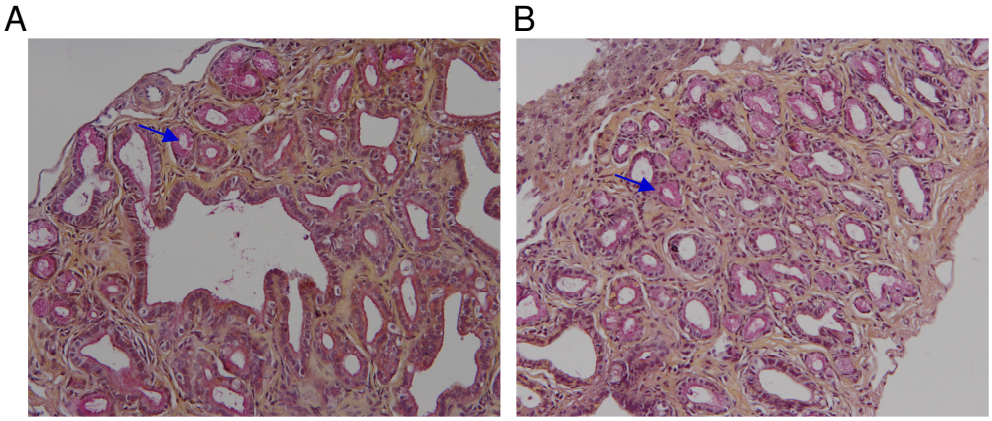


Figure 3-5. Effects of *Tgr5* deficiency on mucin production in the gallbladder.
A, Mucins staining was performed on WT gallbladders. Mucins are stained pink as indicated with arrow. **B,** Mucin staining in *Tgr5*$^{-/-}$ gallbladders did not reveal deficiency in mucin in the gallbladder epithelium. Representative pictures are shown.

Mucin staining in gallbladder tissue sections prepared from WT and *Tgr5*$^{-/-}$ mice did not show significant difference (Figure 3-5), indicating that TGR5 is not required for mucin secretion. However, a quantitative comparison should be done by comparing mucin protein concentration in the gallbladder bile from WT and *Tgr5*$^{-/-}$mice. Any difference could directly reveal any role of mucins in the CGD in these animals. In addition, we detected TGR5 expression in Caco-2 and HT-29 cells. These cell lines, together with primary gallbladder epithelial cells can be used to as cell-based assay for mucin secretions (Klinkspoor, Tytgat *et al.* 1996; Shekels, Lyftogt *et al.* 1996; Klinkspoor, Mok *et al.* 1999).

Perspectives

In addition to facilitating digestion and absorption of dietary fats, the multiple roles of bile acid in the cholesterol, triglyceride and glucose metabolism have been revealed since the discovery of the agonist activity of bile acids on nuclear receptors including FXR, PXR and VDR. The more recent discovery of bile acids as TGR5 ligands on the cell membrane is further increasing our appreciation of the complexity of bile biology as we investigate the phenotypes of *Tgr5*$^{-/-}$ mice and discover the physiological impact of bile acid-dependent TGR5 activation. Table 3-1 summarizes the many important roles of TGR5 in different organs as suggested by recent publications. Because TGR5 agonism is considered a promising therapy for obesity and type 2 diabetes since TGR5 agonism stimulates energy metabolism and lowers serum glucose (Watanabe, Houten *et al.* 2006; Thomas, Gioiello *et al.* 2009), it will be important to assess the impact of its activation in metabolism as TGR5 agonists are developed as drugs.

In this dissertation, we present a role of TGR5 in stimulating gallbladder smooth muscle relaxation and filling with support from our *in vivo* and *ex vivo* experiments. This is consistent with the high expression level of TGR5 in the gallbladder epithelium. We also showed that TGR5 agonist increases cAMP concentration in gallbladder tissues in a TGR5 dependent manner. Work from our laboratory (Choi, Moschetta *et al.* 2006) has shown that the intestinal hormone FGF15/19 regulates gallbladder filling and activates cAMP production. It is thus possible that TGR5 may act in concert with FGF15/19

Liver	Expressed in Kupffer cells, might mediate immunosuppressive effects of bile acids (Keitel, Donner *et al.* 2008); expressed in sinusoidal endothelial cells; induces NO production in sinusoidal endothelial cells and might be involved in vasodilation (Keitel, Reinehr *et al.* 2007); Expressed in cholangiocytes (Keitel, Ullmer *et al.* 2010); might activate CFTR, induces chloride secretion and activates bile secretion (our data and Keitel, Cupisti *et al.* 2009). Mutations associated with primary sclerosing cholangitis (Hov, Keitel *et al.* 2010).
Gallbladder	Expressed in the epithelial cells of the gallbladder (our data Vassileva, Golovko *et al.* 2006; Keitel, Cupisti *et al.* 2009). Relaxes gallbladder smooth muscle (Our data and Lavoie, Balemba *et al.* 2010); activates K_{ATP} channels (Lavoie, Balemba *et al.* 2010) and promotes gallstone formation (Vassileva, Golovko *et al.* 2006).
Intestine	Induces GLP-1 secretion and improves insulin sensitivity (Katsuma, Hirasawa *et al.* 2005; Watanabe, Houten *et al.* 2006; Sato, Genet *et al.* 2007; Thomas, Gioiello *et al.* 2009); expressed in enteric ganglia of the mouse stomach and small and large intestine, and in the muscularis externa and mucosa of the small intestine; regulates intestinal motility (Poole, Godfrey *et al.* 2010).
Immune Cells	Immunomodulation; suppresses cytokine production in macrophages (Kawamata, Fujii *et al.* 2003; Keitel, Reinehr *et al.* 2007; Sato, Genet *et al.* 2007; Keitel, Donner *et al.* 2008).
Adipose Tissue & Skeletal Muscle	Energy expenditure through thyroid hormone signaling (Watanabe, Houten *et al.* 2006).
Brain	Expression in astrocytes and neurons; its activation increases intracellular cAMP, Ca^{2+} and reactive oxygen species; downregulated by neurosteroids; Implications for the pathogenesis of hepatic encephalopathy (Keitel, Gorg *et al.* 2010).

Table 3-1. Functions of TGR5 in key expressing tissues as reported in recent publications.

pathway in signaling the gallbladder to fill, although our *in vivo* data suggest neither TGR5 nor FGF15/19 is required for the relaxation effect of the other pathway. Based on our findings, we suggest a two-pronged "distal/proximal" signaling mechanism for controlling gallbladder filling. FGF15/19, which is induced by bile acids in the ileum, represents a distal hormonal signal that effectively "primes" the gallbladder for refilling after a meal. TGR5, which is present in the gallbladder epithelium, provides a local mechanism to respond to the increased bile acids concentration as bile is secreted from the liver and concentrated in the gallbladder. In this regard, it is intriguing that TGR5 induces chloride secretion from the gallbladder epithelium via CFTR (Keitel, Cupisti *et al.* 2009), which may provide contribute to gallbladder filling. Future experiments will be conducted to further defining the relationship between TGR5 and FGF15/19 and understanding their respective roles in regulating gallbladder motility.

While this manuscript is in preparation, Lavoie *et al.* (2010) presented *ex vivo* data supporting a role of TGR5 in activating gallbladder smooth muscle relaxation via cAMP-PKA-K_{ATP} pathway. Their report of TGR5 localization in the gallbladder smooth muscle, however, is inconsistent with our observation and previous reports (Vassileva, Golovko *et al.* 2006; Keitel, Cupisti *et al.* 2009). Thus, further investigation of TGR5 localization will be important to further understand TGR5 action in the gallbladder.

It has been previously shown that *Tgr5*$^{-/-}$ mice are resistant to diet-induced cholesterol gallstone formation which was attributed to increased cyp7a1 expression in the liver, increased phospholipid concentration in the bile, and a corresponding reduction in the cholesterol saturation index (Vassileva, Golovko *et al.* 2006). In this dissertation

we report that the bile acid pool size and composition in *Tgr5*$^{-/-}$ mice are significantly altered, but TGR5 is not directly involved in the feedback regulation of bile acid biosynthesis. We also presented preliminary data on dysregulated bile acid transporters in the ileum of *Tgr5*$^{-/-}$ mice, which suggests that TGR5 might play an important role in regulating bile acid absorption in the intestine and can explain the altered bile acid pool size and the increased tCA/tMCA ratio found in the bile acid pool of *Tgr5*$^{-/-}$ mice. One remaining problem is that the increased tCA/tMCA ratio suggests increased hydrophobicity in the bile of these animals. This is the opposite of what is expected considering the CGD resistant phenotype in *Tgr5*$^{-/-}$ mice, because increased bile hydrophobicity is associated with increased gallstone formation. While it is possible that the decreased gallbladder filling in the *Tgr5*$^{-/-}$ mice might contribute to the reduced gallstone formation in these animals, the role of TGR5 in the pathogenesis of gallstone formation will need to be further examined.

BIBLIOGRAPHY

Bookout, A. L., C. L. Cummins, *et al.* (2006). "High-throughput real-time quantitative reverse transcription PCR." Curr Protoc Mol Biol Chapter 15: Unit 15 18.

Lee, Y. K., D. R. Schmidt, *et al.* (2008). "Liver receptor homolog-1 regulates bile acid homeostasis but is not essential for feedback regulation of bile acid synthesis." Mol Endocrinol 22(6): 1345-1356.

Turley, S. D., M. Schwarz, *et al.* (1998). "Gender-related differences in bile acid and sterol metabolism in outbred CD-1 mice fed low- and high-cholesterol diets." Hepatology 28(4): 1088-1094.

Vassileva, G., A. Golovko, *et al.* (2006). "Targeted deletion of Gpbar1 protects mice from cholesterol gallstone formation." Biochem J 398(3): 423-430.

Abu-Hamdah, R., A. Rabiee, *et al.* (2009). "Clinical review: The extrapancreatic effects of glucagon-like peptide-1 and related peptides." J Clin Endocrinol Metab **94**(6): 1843-1852.

Adams, D. H. (1996). "Biliary epithelial cells: innocent victims or active participants in immune-mediated liver disease?" J Lab Clin Med **128**(6): 528-530.

Alpini, G., S. Glaser, *et al.* (1997). "Bile acids stimulate proliferative and secretory events in large but not small cholangiocytes." Am J Physiol **273**(2 Pt 1): G518-529.

Alrefai, W. A. and R. K. Gill (2007). "Bile acid transporters: structure, function, regulation and pathophysiological implications." Pharm Res **24**(10): 1803-1823.

Amdrup, B. M. and C. A. Griffith (1970). "The effects of vagotomy upon biliary function in dogs." J Surg Res **10**(5): 209-212.

Amelsberg, A., C. Jochims, *et al.* (1999). "Evidence for an anion exchange mechanism for uptake of conjugated bile acid from the rat jejunum." Am J Physiol **276**(3 Pt 1): G737-742.

Amer, M. S. (1972). "Studies with cholecystokinin in vitro. 3. Mechanism of the effect on the isolated rabbit gall bladder strips." J Pharmacol Exp Ther **183**(3): 527-534.

Ananthanarayanan, M., N. Balasubramanian, *et al.* (2001). "Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor." J Biol Chem **276**(31): 28857-28865.

Angelin, B., I. Bjorkhem, *et al.* (1982). "Hepatic uptake of bile acids in man. Fasting and postprandial concentrations of individual bile acids in portal venous and systemic blood serum." J Clin Invest **70**(4): 724-731.

Anwer, M. S. (2004). "Cellular regulation of hepatic bile acid transport in health and cholestasis." Hepatology **39**(3): 581-590.

Barrett, K., F. K. Ghishan, *et al.* (2006). Gastrointestinal Physiology, Academic Press.
Bianco, A. C. and B. W. Kim (2006). "Deiodinases: implications of the local control of thyroid hormone action." J Clin Invest **116**(10): 2571-2579.
Bijvelds, M. J., H. Jorna, *et al.* (2005). "Activation of CFTR by ASBT-mediated bile salt absorption." Am J Physiol Gastrointest Liver Physiol **289**(5): G870-879.
Bookout, A. L., C. L. Cummins, *et al.* (2006). "High-throughput real-time quantitative reverse transcription PCR." Curr Protoc Mol Biol **Chapter 15**: Unit 15 18.
Brubaker, P. L. (2010). "Minireview: update on incretin biology: focus on glucagon-like peptide-1." Endocrinology **151**(5): 1984-1989.
Brufau, G., M. J. Bahr, *et al.* (2010). "Plasma bile acids are not associated with energy metabolism in humans." Nutr Metab (Lond) **7**: 73.
Buchan, A. M., J. M. Polak, *et al.* (1978). "Electron immunohistochemical evidence for the human intestinal I cell as the source of CCK." Gut **19**(5): 403-407.
Cai, S. Y. and J. L. Boyer (2006). "FXR: a target for cholestatic syndromes?" Expert Opin Ther Targets **10**(3): 409-421.
Carey, M. C. (1978). "Critical tables for calculating the cholesterol saturation of native bile." J Lipid Res **19**(8): 945-955.
Cheng, K. and J. P. Raufman (2005). "Bile acid-induced proliferation of a human colon cancer cell line is mediated by transactivation of epidermal growth factor receptors." Biochem Pharmacol **70**(7): 1035-1047.
Chiang, J. Y. (2002). "Bile acid regulation of gene expression: roles of nuclear hormone receptors." Endocr Rev **23**(4): 443-463.
Cho, W. K. (2000). Bile ductal secretion and its regulation. Gallbladder and Biliary Tract Diseases
N. H. Afdhal. New York, Marcel Dekker, Inc
99-125.
Choi, M., A. Moschetta, *et al.* (2006). "Identification of a hormonal basis for gallbladder filling." Nat Med **12**(11): 1253-1255.
Chuang, S. C., S. H. Juo, *et al.* (2010). "Multiple mucin genes polymorphisms are associated with gallstone disease in Chinese Men." Clin Chim Acta.
Cohn, J. A., T. V. Strong, *et al.* (1993). "Localization of the cystic fibrosis transmembrane conductance regulator in human bile duct epithelial cells." Gastroenterology **105**(6): 1857-1864.
Dancygier, H. (2009). Clinical Hepatology: Principles and Practice of Hepatobiliary Diseases, Springer; 1 edition
Davenport, H. W. (1966). Physiology of the Digestive Tract. Chicago, Year Book.

Dawson, P. A., J. Haywood, *et al.* (2003). "Targeted deletion of the ileal bile acid transporter eliminates enterohepatic cycling of bile acids in mice." J Biol Chem **278**(36): 33920-33927.
Dawson, P. A., T. Lan, *et al.* (2009). "Bile acid transporters." J Lipid Res **50**(12): 2340-2357.
de la Porte, P. L., N. Domingo, *et al.* (1996). "Distinct immuno-localization of mucin and other biliary proteins in human cholesterol gallstones." J Hepatol **25**(3): 339-348.
Diamond, J. M. (1964). "The Mechanism of Isotonic Water Transport." J Gen Physiol **48**: 15-42.
Dranoff, J. A., M. McClure, *et al.* (1999). "Short-term regulation of bile acid uptake by microfilament-dependent translocation of rat ntcp to the plasma membrane." Hepatology **30**(1): 223-229.
Drucker, D. J. (2006). "The biology of incretin hormones." Cell Metab **3**(3): 153-165.
Duane, W. C. and K. C. Hanson (1978). "Role of gallbladder emptying and small bowel transit in regulation of bile acid pool size in man." J Lab Clin Med **92**(6): 858-872.
Esteller, A. (2008). "Physiology of bile secretion." World J Gastroenterol **14**(37): 5641-5649.
Fickert, P., A. Fuchsbichler, *et al.* (2004). "Regurgitation of bile acids from leaky bile ducts causes sclerosing cholangitis in Mdr2 (Abcb4) knockout mice." Gastroenterology **127**(1): 261-274.
Fisher, R. S., E. Rock, *et al.* (1986). "Gallbladder emptying response to sham feeding in humans." Gastroenterology **90**(6): 1854-1857.
Forker, E. L. (1977). "Mechanisms of hepatic bile formation." Annu Rev Physiol **39**: 323-347.
Forstner, G., Y. Zhang, *et al.* (1993). "Mucin secretion by T84 cells: stimulation by PKC, Ca2+, and a protein kinase activated by Ca2+ ionophore." Am J Physiol **264**(6 Pt 1): G1096-1102.
Forstner, G., Y. Zhang, *et al.* (1994). "Regulation of mucin secretion in T84 adenocarcinoma cells by forskolin: relationship to Ca2+ and PKC." Am J Physiol **266**(4 Pt 1): G606-612.
Gatmaitan, Z. C., A. T. Nies, *et al.* (1997). "Regulation and translocation of ATP-dependent apical membrane proteins in rat liver." Am J Physiol **272**(5 Pt 1): G1041-1049.
Gerloff, T., B. Stieger, *et al.* (1998). "The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver." J Biol Chem **273**(16): 10046-10050.
Germain, P., B. Staels, *et al.* (2006). "Overview of nomenclature of nuclear receptors." Pharmacol Rev **58**(4): 685-704.

Goodwin, B., K. C. Gauthier, *et al.* (2003). "Identification of bile acid precursors as endogenous ligands for the nuclear xenobiotic pregnane X receptor." Proc Natl Acad Sci U S A **100**(1): 223-228.

Goodwin, B., S. A. Jones, *et al.* (2000). "A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis." Mol Cell **6**(3): 517-526.

Grober, J., I. Zaghini, *et al.* (1999). "Identification of a bile acid-responsive element in the human ileal bile acid-binding protein gene. Involvement of the farnesoid X receptor/9-cis-retinoic acid receptor heterodimer." J Biol Chem **274**(42): 29749-29754.

Hay, D. W. and M. C. Carey (1990). "Pathophysiology and pathogenesis of cholesterol gallstone formation." Semin Liver Dis **10**(3): 159-170.

Hedner, P. (1970). "Effect of the C-terminal octapeptide of cholecystokinin on guinea pig ileum and gall-bladder in vitro." Acta Physiol Scand **78**(2): 232-235.

Hoffman, A. F. (1994). Intestinal absorption of bile acids and biliary constituents: the intestinal components of the enterohepatic circulation and the integrated system. Physiology of the gastrointestinal tract,. L. R. Johnson. New York, Raven Press**:** pp. 1845–1865.

Hofmann, A. F. (2007). "Biliary secretion and excretion in health and disease: current concepts." Ann Hepatol **6**(1): 15-27.

Holt, J. A., G. Luo, *et al.* (2003). "Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis." Genes Dev **17**(13): 1581-1591.

Hopman, W. P., J. B. Jansen, *et al.* (1987). "Cephalic stimulation of gallbladder contraction in humans: role of cholecystokinin and the cholinergic system." Digestion **38**(4): 197-203.

Hopwood, D. and P. E. Ross (1997). "Biochemical and morphological correlations in human gallbladder with reference to membrane permeability." Microsc Res Tech **38**(6): 631-642.

Houten, S. M. (2006). "Homing in on bile acid physiology." Cell Metab **4**(6): 423-424.

Hov, J. R., V. Keitel, *et al.* (2010). "Mutational characterization of the bile acid receptor TGR5 in primary sclerosing cholangitis." PLoS One **5**(8): e12403.

Hwang, S. T., N. L. Urizar, *et al.* (2002). "Bile acids regulate the ontogenic expression of ileal bile acid binding protein in the rat via the farnesoid X receptor." Gastroenterology **122**(5): 1483-1492.

Ikemoto, S., M. Takahashi, *et al.* (1997). "Cholate inhibits high-fat diet-induced hyperglycemia and obesity with acyl-CoA synthetase mRNA decrease." Am J Physiol **273**(1 Pt 1): E37-45.

Inagaki, T., M. Choi, *et al.* (2005). "Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis." Cell Metab **2**(4): 217-225.
Ivy, A. C. and E. Oldberg (1928). "A Hormone Mechanism for Gall-Bladder Contraction and Evacuation." Am J Physiology(86): 599-613.
Jazrawi, R. P. (2000). Normal Gallbladder Motor Function. Gallbladder and Biliary Tract Disease. N. H. Afdhal. New York, Marcel Dekker**:** 251-266.
Jazrawi, R. P. and T. C. Northfield (1986). "Effects of a pharmacological dose of cholecystokinin on bile acid kinetics and biliary cholesterol saturation in man." Gut **27**(4): 355-362.
Jean-Louis, S., S. Akare, *et al.* (2006). "Deoxycholic acid induces intracellular signaling through membrane perturbations." J Biol Chem **281**(21): 14948-14960.
Kalaany, N. Y. and D. J. Mangelsdorf (2006). "LXRS and FXR: the yin and yang of cholesterol and fat metabolism." Annu Rev Physiol **68**: 159-191.
Katsuma, S., A. Hirasawa, *et al.* (2005). "Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1." Biochem Biophys Res Commun **329**(1): 386-390.
Kawamata, Y., R. Fujii, *et al.* (2003). "A G protein-coupled receptor responsive to bile acids." J Biol Chem **278**(11): 9435-9440.
Keitel, V., K. Cupisti, *et al.* (2009). "The membrane-bound bile acid receptor TGR5 is localized in the epithelium of human gallbladders." Hepatology **50**(3): 861-870.
Keitel, V., M. Donner, *et al.* (2008). "Expression and function of the bile acid receptor TGR5 in Kupffer cells." Biochem Biophys Res Commun **372**(1): 78-84.
Keitel, V., B. Gorg, *et al.* (2010). "The bile acid receptor TGR5 (Gpbar-1) acts as a neurosteroid receptor in brain." Glia **58**(15): 1794-1805.
Keitel, V., R. Reinehr, *et al.* (2007). "The G-protein coupled bile salt receptor TGR5 is expressed in liver sinusoidal endothelial cells." Hepatology **45**(3): 695-704.
Keitel, V., C. Ullmer, *et al.* (2010). "The membrane-bound bile acid receptor TGR5 (Gpbar-1) is localized in the primary cilium of cholangiocytes." Biol Chem **391**(7): 785-789.
King, J. E. and L. J. Schoenfield (1972). "Lithocholic acid, cholestasis, and liver disease." Mayo Clin Proc **47**(10): 725-730.
Kipp, H., N. Pichetshote, *et al.* (2001). "Transporters on demand: intrahepatic pools of canalicular ATP binding cassette transporters in rat liver." J Biol Chem **276**(10): 7218-7224.

Kliewer, S. A. and T. M. Willson (2002). "Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor." J Lipid Res **43**(3): 359-364.

Klinkspoor, J. H., R. Kuver, *et al.* (1995). "Model bile and bile salts accelerate mucin secretion by cultured dog gallbladder epithelial cells." Gastroenterology **109**(1): 264-274.

Klinkspoor, J. H. and S. P. Lee (2000). Gallbladder Mucosal Function. Gallbladder and Biliary Tract Diseases. N. H. Afdhal. New York, Marcel Dekker, Inc**:** 39-64.

Klinkspoor, J. H., K. S. Mok, *et al.* (1999). "Mucin secretion by the human colon cell line LS174T is regulated by bile salts." Glycobiology **9**(1): 13-19.

Klinkspoor, J. H., G. N. Tytgat, *et al.* (1996). "Mechanism of bile salt-induced mucin secretion by cultured dog gallbladder epithelial cells." Biochem J **316 (Pt 3)**: 873-877.

LaMont, J. T., B. S. Turner, *et al.* (1983). "Arachidonic acid stimulates mucin secretion in prairie dog gallbladder." Am J Physiol **245**(1): G92-98.

LaMorte, W. W., J. T. LaMont, *et al.* (1986). "Gallbladder prostaglandins and lysophospholipids as mediators of mucin secretion during cholelithiasis." Am J Physiol **251**(5 Pt 1): G701-709.

Landrier, J. F., J. Grober, *et al.* (2002). "Regulation of the ileal bile acid-binding protein gene: an approach to determine its physiological function(s)." Mol Cell Biochem **239**(1-2): 149-155.

Lanzini, A. and F. Lanzarotto (2000). "Review article: the 'mechanical pumps' and the enterohepatic circulation of bile acids--defects in coeliac disease." Aliment Pharmacol Ther **14 Suppl 2**: 58-61.

Lavoie, B., O. B. Balemba, *et al.* "Hydrophobic bile salts inhibit gallbladder smooth muscle function via stimulation of GPBAR1 receptors and activation of KATP channels." J Physiol **588**(Pt 17): 3295-3305.

Lavoie, B., O. B. Balemba, *et al.* (2010). "Hydrophobic bile salts inhibit gallbladder smooth muscle function via stimulation of GPBAR1 receptors and activation of KATP channels." J Physiol **588**(Pt 17): 3295-3305.

Lee, J. H. K. a. S. P. (2000). Gallbladder Mucosal function. Gallbladder and Biliary Tract Diseases. N. H. Afdhal. New York, Marcel Dekker**:** 21-38.

Lee, S. P., J. T. LaMont, *et al.* (1981). "Role of gallbladder mucus hypersecretion in the evolution of cholesterol gallstones." J Clin Invest **67**(6): 1712-1723.

Lee, Y. K., D. R. Schmidt, *et al.* (2008). "Liver receptor homolog-1 regulates bile acid homeostasis but is not essential for feedback regulation of bile acid synthesis." Mol Endocrinol **22**(6): 1345-1356.

Liddle, R. A., B. J. Gertz, *et al.* (1989). "Effects of a novel cholecystokinin (CCK) receptor antagonist, MK-329, on gallbladder contraction and gastric

emptying in humans. Implications for the physiology of CCK." J Clin Invest **84**(4): 1220-1225.
Liddle, R. A., B. J. Gertz, *et al.* (1990). "Regulation of pancreatic endocrine function by cholecystokinin: studies with MK-329, a nonpeptide cholecystokinin receptor antagonist." J Clin Endocrinol Metab **70**(5): 1312-1318.
Lindblad, L., K. Lundholm, *et al.* (1977). "Bile acid concentrations in systemic and portal serum in presumably normal man and in cholestatic and cirrhotic conditions." Scand J Gastroenterol **12**(4): 395-400.
Low-Beer, T. S., K. W. Heaton, *et al.* (1971). "Gallbladder inertia and sluggish enterohepatic circulation of bile-salts in coeliac disease." Lancet **1**(7707): 991-994.
Lu, T. T., M. Makishima, *et al.* (2000). "Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors." Mol Cell **6**(3): 507-515.
Ma, K., P. K. Saha, *et al.* (2006). "Farnesoid X receptor is essential for normal glucose homeostasis." J Clin Invest **116**(4): 1102-1109.
MacPherson, B. R. and R. S. Pemsingh (1997). "Ground squirrel model for cholelithiasis: role of epithelial glycoproteins." Microsc Res Tech **39**(1): 39-55.
Maki, T., T. Matsushiro, *et al.* (1971). "Role of sulfated glycoprotein in gallstone formation." Surg Gynecol Obstet **132**(5): 846-854.
Makishima, M., T. T. Lu, *et al.* (2002). "Vitamin D receptor as an intestinal bile acid sensor." Science **296**(5571): 1313-1316.
Makishima, M., A. Y. Okamoto, *et al.* (1999). "Identification of a nuclear receptor for bile acids." Science **284**(5418): 1362-1365.
Margolis, R. N. and S. Christakos (2010). "The nuclear receptor superfamily of steroid hormones and vitamin D gene regulation. An update." Ann N Y Acad Sci **1192**: 208-214.
Marinelli, R. A., L. D. Pham, *et al.* (2000). "Expression of aquaporin-4 water channels in rat cholangiocytes." Hepatology **31**(6): 1313-1317.
Marinelli, R. A., P. S. Tietz, *et al.* (1999). "Secretin induces the apical insertion of aquaporin-1 water channels in rat cholangiocytes." Am J Physiol **276**(1 Pt 1): G280-286.
Martinez-Anso, E., J. E. Castillo, *et al.* (1994). "Immunohistochemical detection of chloride/bicarbonate anion exchangers in human liver." Hepatology **19**(6): 1400-1406.
Maruyama, T., Y. Miyamoto, *et al.* (2002). "Identification of membrane-type receptor for bile acids (M-BAR)." Biochem Biophys Res Commun **298**(5): 714-719.

Maruyama, T., K. Tanaka, *et al.* (2006). "Targeted disruption of G protein-coupled bile acid receptor 1 (Gpbar1/M-Bar) in mice." J Endocrinol **191**(1): 197-205.
Marzio, L., M. Neri, *et al.* (1988). "Gallbladder contraction and its relationship to interdigestive duodenal motor activity in normal human subjects." Dig Dis Sci **33**(5): 540-544.
Mawe, G. M. (1991). "The role of cholecystokinin in ganglionic transmission in the guinea-pig gall-bladder." J Physiol **439**: 89-102.
McCool, D. J., M. A. Marcon, *et al.* (1990). "The T84 human colonic adenocarcinoma cell line produces mucin in culture and releases it in response to various secretagogues." Biochem J **267**(2): 491-500.
Meier, P. J. and B. Stieger (2000). "Molecular Mechanisms in Bile Formation." News Physiol Sci **15**: 89-93.
Mennone, A., D. Biemesderfer, *et al.* (2001). "Role of sodium/hydrogen exchanger isoform NHE3 in fluid secretion and absorption in mouse and rat cholangiocytes." Am J Physiol Gastrointest Liver Physiol **280**(2): G247-254.
Merchant, N. B., C. M. Rogers, *et al.* (2005). "Ligand-dependent activation of the epidermal growth factor receptor by secondary bile acids in polarizing colon cancer cells." Surgery **138**(3): 415-421.
Moschetta, A., A. L. Bookout, *et al.* (2004). "Prevention of cholesterol gallstone disease by FXR agonists in a mouse model." Nat Med **10**(12): 1352-1358.
Mukhopadhayay, S., M. Ananthanarayanan, *et al.* (1997). "cAMP increases liver Na+-taurocholate cotransport by translocating transporter to plasma membranes." Am J Physiol **273**(4 Pt 1): G842-848.
Mussig, K., H. Staiger, *et al.* (2009). "Preliminary report: genetic variation within the GPBAR1 gene is not associated with metabolic traits in white subjects at an increased risk for type 2 diabetes mellitus." Metabolism **58**(12): 1809-1811.
Nakahara, M., N. Furuya, *et al.* (2005). "Ileal bile acid-binding protein, functionally associated with the farnesoid X receptor or the ileal bile acid transporter, regulates bile acid activity in the small intestine." J Biol Chem **280**(51): 42283-42289.
Northfield, T. C., H. J. Ahmed, *et al.* (2000). Bile acids in hepatobiliary disease, Springer.
O'Grady, S. M., P. J. Wolters, *et al.* (1989). "Regulation of ion transport in porcine gallbladder: effects of VIP and norepinephrine." Am J Physiol **257**(1 Pt 1): C52-57.
Offner, G. D. (2000). Gallbladder Mucin. Gallbladder and Biliary Tract Diseases. N. H. Afdhal. New York, Marcel Dekker**:** 211.

Otsuki, M. (2000). "Pathophysiological role of cholecystokinin in humans." J Gastroenterol Hepatol **15 Suppl**: D71-83.
Pandak, W. M., P. Bohdan, *et al.* (2001). "Expression of sterol 12alpha-hydroxylase alters bile acid pool composition in primary rat hepatocytes and in vivo." Gastroenterology **120**(7): 1801-1809.
Pauletzki, J. G., K. A. Sharkey, *et al.* (1993). "Involvement of L-arginine-nitric oxide pathways in neural relaxation of the sphincter of Oddi." Eur J Pharmacol **232**(2-3): 263-270.
Pellicciari, R., A. Gioiello, *et al.* (2009). "Discovery of 6alpha-ethyl-23(S)-methylcholic acid (S-EMCA, INT-777) as a potent and selective agonist for the TGR5 receptor, a novel target for diabesity." J Med Chem **52**(24): 7958-7961.
Pemsingh, R. S., B. R. MacPherson, *et al.* (1987). "Mucus hypersecretion in the gallbladder epithelium of ground squirrels fed a lithogenic diet for the induction of cholesterol gallstones." Hepatology **7**(6): 1267-1271.
Pennington, C. R., P. E. Ross, *et al.* (1982). "Influence of the gallbladder on serum bile acids." J Clin Pathol **35**(7): 754-756.
Petersen, K. U. and L. Reuss (1983). "Cyclic AMP-induced chloride permeability in the apical membrane of Necturus gallbladder epithelium." J Gen Physiol **81**(5): 705-729.
Petersen, K. U., F. Wehner, *et al.* (1985). "Na/H exchange at the apical membrane of guinea-pig gallbladder epithelium: properties and inhibition by cyclic AMP." Pflugers Arch **405 Suppl 1**: S115-120.
Plass, J. R., O. Mol, *et al.* (2002). "Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump." Hepatology **35**(3): 589-596.
Poole, D. P., C. Godfrey, *et al.* (2010). "Expression and function of the bile acid receptor GpBAR1 (TGR5) in the murine enteric nervous system." Neurogastroenterol Motil **22**(7): 814-825, e227-818.
Portincasa, P., A. Di Ciaula, *et al.* (2008). "Coordinate regulation of gallbladder motor function in the gut-liver axis." Hepatology **47**(6): 2112-2126.
Qiao, L., E. Studer, *et al.* (2001). "Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis." Mol Biol Cell **12**(9): 2629-2645.
Repa, J. J. and D. J. Mangelsdorf (1999). "Nuclear receptor regulation of cholesterol and bile acid metabolism." Curr Opin Biotechnol **10**(6): 557-563.

Reuss, L. (1987). "Cyclic AMP inhibits Cl-/HCO3- exchange at the apical membrane of Necturus gallbladder epithelium." J Gen Physiol **90**(2): 173-196.
Reuss, L. (1991). Salt and water transport by gallbladder epithelium. Handbook of Physiology: The Gastrointestinal System. M. Bethsda, American Physiological Society**:** p302-322.
Reuss, L., Y. Segal, *et al.* (1991). "Regulation of ion transport across gallbladder epithelium." Annu Rev Physiol **53**: 361-373.
Rizzo, G., D. Passeri, *et al.* (2010). "Functional characterization of the semisynthetic bile acid derivative INT-767, a dual farnesoid X receptor and TGR5 agonist." Mol Pharmacol **78**(4): 617-630.
Roda, E., R. Aldini, *et al.* (1978). "Enterohepatic circulation of bile acids after cholecystectomy." Gut **19**(7): 640-649.
Roelofsen, H., C. J. Soroka, *et al.* (1998). "Cyclic AMP stimulates sorting of the canalicular organic anion transporter (Mrp2/cMoat) to the apical domain in hepatocyte couplets." J Cell Sci **111 (Pt 8)**: 1137-1145.
Romer, A. S. and T. S. Parsons (1977). Vertebrate Body Philadelphia, PA, W.B. Saunders Company.
Russell, D. W. (2003). "The enzymes, regulation, and genetics of bile acid synthesis." Annu Rev Biochem **72**: 137-174.
Russell, D. W. (2009). "Fifty years of advances in bile acid synthesis and metabolism." J Lipid Res **50 Suppl**: S120-125.
Rutishauser, S. (1978). "Effect of bile salts on the motor activity of the guinea-pig gallbladder in vitro. ." Q J exp physiol cong med sci (63): 265-276.
Sato, H., C. Genet, *et al.* (2007). "Anti-hyperglycemic activity of a TGR5 agonist isolated from Olea europaea." Biochem Biophys Res Commun **362**(4): 793-798.
Schjoldager, B. T. (1994). "Role of CCK in gallbladder function." Ann N Y Acad Sci **713**: 207-218.
Schmidt, W. E., W. Creutzfeldt, *et al.* (1991). "Role of CCK in regulation of pancreaticobiliary functions and GI motility in humans: effects of loxiglumide." Am J Physiol **260**(2 Pt 1): G197-206.
Scoazec, J. Y., A. F. Bringuier, *et al.* (1997). "The plasma membrane polarity of human biliary epithelial cells: in situ immunohistochemical analysis and functional implications." J Hepatol **26**(3): 543-553.
Sellers, L. A., A. Allen, *et al.* (1988). "Mucus glycoprotein gels. Role of glycoprotein polymeric structure and carbohydrate side-chains in gel-formation." Carbohydr Res **178**: 93-110.
Shaffer, E. A., P. McOrmond, *et al.* (1980). "Quantitative cholescintigraphy: assessment of gallbladder filling and emptying and duodenogastric reflux." Gastroenterology **79**(5 Pt 1): 899-906.

Sheehan, J. K., K. Oates, *et al.* (1986). "Electron microscopy of cervical, gastric and bronchial mucus glycoproteins." Biochem J **239**(1): 147-153.
Shekels, L. L., C. T. Lyftogt, *et al.* (1996). "Bile acid-induced alterations of mucin production in differentiated human colon cancer cell lines." Int J Biochem Cell Biol **28**(2): 193-201.
Smit, J. J., A. H. Schinkel, *et al.* (1993). "Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease." Cell **75**(3): 451-462.
Smith, B. F. (1987). "Human gallbladder mucin binds biliary lipids and promotes cholesterol crystal nucleation in model bile." J Lipid Res **28**(9): 1088-1097.
St-Pierre, M. V., G. A. Kullak-Ublick, *et al.* (2001). "Transport of bile acids in hepatic and non-hepatic tissues." J Exp Biol **204**(Pt 10): 1673-1686.
Steigerwalt, R. W., I. D. Goldfine, *et al.* (1984). "Characterization of cholecystokinin receptors on bovine gallbladder membranes." Am J Physiol **247**(6 Pt 1): G709-714.
Stelzner, M., S. Somasundaram, *et al.* (2001). "Ileal mucosal bile acid absorption is increased in Cftr knockout mice." BMC Gastroenterol **1**: 10.
Strazzabosco, M. (1997). "New insights into cholangiocyte physiology." J Hepatol **27**(5): 945-952.
Strazzabosco, M., L. Fabris, *et al.* (2005). "Pathophysiology of cholangiopathies." J Clin Gastroenterol **39**(4 Suppl 2): S90-S102.
Strous, G. J. and J. Dekker (1992). "Mucin-type glycoproteins." Crit Rev Biochem Mol Biol **27**(1-2): 57-92.
Svenberg, T., N. D. Christofides, *et al.* (1982). "Interdigestive biliary output in man: relationship to fluctuations in plasma motilin and effect of atropine." Gut **23**(12): 1024-1028.
Tanaka, M. (2010). "Function and dysfunction of the sphincter of Oddi." Dig Surg **27**(2): 94-99.
Thomas, C., A. Gioiello, *et al.* (2009). "TGR5-mediated bile acid sensing controls glucose homeostasis." Cell Metab **10**(3): 167-177.
Tomlinson, E., L. Fu, *et al.* (2002). "Transgenic mice expressing human fibroblast growth factor-19 display increased metabolic rate and decreased adiposity." Endocrinology **143**(5): 1741-1747.
Toouli, J. and A. Craig (2000). "Sphincter of Oddi function and dysfunction." Can J Gastroenterol **14**(5): 411-419.
Toouli, J., W. J. Hogan, *et al.* (1982). "Action of cholecystokinin-octapeptide on sphincter of Oddi basal pressure and phasic wave activity in humans." Surgery **92**(3): 497-503.

Tooull, J. and M. Bhandari (2007). Anatomy and Physiology of the Biliary Tree and Gallbladder. Diseases of the Gallbladder and Bile Ducts: Diagnosis and Treatment. P.-A. Clavien and J. Baillie, Blackwell Publishing Ltd.

Torsoli, A., E. Corazziari, *et al.* (1986). "Frequencies and cyclical pattern of the human sphincter of Oddi phasic activity." Gut **27**(4): 363-369.

Turley, S. D., M. Schwarz, *et al.* (1998). "Gender-related differences in bile acid and sterol metabolism in outbred CD-1 mice fed low- and high-cholesterol diets." Hepatology **28**(4): 1088-1094.

van Erpecum, K. J. (2005). "Biliary lipids, water and cholesterol gallstones." Biol Cell **97**(11): 815-822.

Van Erpecum, K. J. and G. P. Van Berge-Henegouwen (1999). "Gallstones: an intestinal disease?" Gut **44**(3): 435-438.

van Ooteghem, N. A., A. Moschetta, *et al.* (2002). "Intraduodenal conjugated bile salts exert negative feedback control on gall bladder emptying in the fasting state without affecting cholecystokinin release or antroduodenal motility." Gut **50**(5): 669-674.

Vassileva, G., A. Golovko, *et al.* (2006). "Targeted deletion of Gpbar1 protects mice from cholesterol gallstone formation." Biochem J **398**(3): 423-430.

Wang, D. Q. and M. C. Carey (1996). "Complete mapping of crystallization pathways during cholesterol precipitation from model bile: influence of physical-chemical variables of pathophysiologic relevance and identification of a stable liquid crystalline state in cold, dilute and hydrophilic bile salt-containing systems." J Lipid Res **37**(3): 606-630.

Wang, D. Q., D. E. Cohen, *et al.* (2009). "Biliary lipids and cholesterol gallstone disease." J Lipid Res **50 Suppl**: S406-411.

Wang, H. H., N. H. Afdhal, *et al.* (2004). "Targeted disruption of the murine mucin gene 1 decreases susceptibility to cholesterol gallstone formation." J Lipid Res **45**(3): 438-447.

Wang, H. H., N. H. Afdhal, *et al.* (2006). "Evidence that gallbladder epithelial mucin enhances cholesterol cholelithogenesis in MUC1 transgenic mice." Gastroenterology **131**(1): 210-222.

Wang, H. H., S. B. Patel, *et al.* (2007). "Quantifying anomalous intestinal sterol uptake, lymphatic transport, and biliary secretion in Abcg8(-/-) mice." Hepatology **45**(4): 998-1006.

Wang, L., J. Liu, *et al.* (2005). "The orphan nuclear receptor SHP regulates PGC-1alpha expression and energy production in brown adipocytes." Cell Metab **2**(4): 227-238.

Wang, R., M. Salem, *et al.* (2001). "Targeted inactivation of sister of P-glycoprotein gene (spgp) in mice results in nonprogressive but persistent intrahepatic cholestasis." Proc Natl Acad Sci U S A **98**(4): 2011-2016.

Watanabe, M., S. M. Houten, *et al.* (2006). "Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation." Nature **439**(7075): 484-489.

Watanabe, M., S. M. Houten, *et al.* (2004). "Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c." J Clin Invest **113**(10): 1408-1418.

Wiley, J. W., T. M. O'Dorisio, *et al.* (1988). "Vasoactive intestinal polypeptide mediates cholecystokinin-induced relaxation of the sphincter of Oddi." J Clin Invest **81**(6): 1920-1924.

Wood, J. R. and J. Svanvik (1983). "Gall-bladder water and electrolyte transport and its regulation." Gut **24**(6): 579-593.

Woods, C. M. and G. T. Saccone (2007). "Neurohormonal regulation of the sphincter of Oddi." Curr Gastroenterol Rep **9**(2): 165-170.

Worthley, C. S., R. A. Baker, *et al.* (1989). "Human fasting and postprandial sphincter of Oddi motility." Br J Surg **76**(7): 709-714.

Yamamura, T., T. Takahashi, *et al.* (1988). "Gallbladder dynamics and plasma cholecystokinin responses after meals, oral water, or sham feeding in healthy subjects." Am J Med Sci **295**(2): 102-107.

Yasuda, H., S. Hirata, *et al.* (2007). "Involvement of membrane-type bile acid receptor M-BAR/TGR5 in bile acid-induced activation of epidermal growth factor receptor and mitogen-activated protein kinases in gastric carcinoma cells." Biochem Biophys Res Commun **354**(1): 154-159.

Yau, W. M., G. M. Makhlouf, *et al.* (1973). "Mode of action of cholecystokinin and related peptides on gallbladder muscle." Gastroenterology **65**(3): 451-456.

Yu, L., J. Li-Hawkins, *et al.* (2002). "Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol." J Clin Invest **110**(5): 671-680.