ENTEROHEPATIC NUCLEAR RECEPTOR SIGNALING DURING PREGNANCY

By

JAMIE ESTHER MOSCOVITZ

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And approved by

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ABSTRACT OF THE DISSERTATION

Enterohepatic Nuclear Receptor Signaling During Pregnancy

By JAMIE ESTHER MOSCOVITZ

Dissertation Director:

Lauren Aleksunes, PharmD, PhD, DABT

Physiological adaptations to the intestine-liver axis during pregnancy alter the disposition of xenobiotics and endobiotics. As a result, some pregnant women experience decreased efficacy or adverse reactions to prescription medications (xenobiotic effect), while others may become susceptible to cholestatic liver disease due to enhanced bile acid levels (endobiotic effect). The purpose of this dissertation research was to characterize molecular adaptations in enterohepatic nuclear receptor signaling and downstream bile acid and xenobiotic disposition pathways that can occur during late pregnancy. In ilea collected from mice in late gestation, an overall down-regulation of Farnesoid X Receptor (Fxr/*Nr1h4*) and Pregnane X Receptor (Pxr/*Nr1i2*) signaling, including target enzyme and transporter expression, was observed. To explore potential mechanisms, the ability of candidate sex hormones to alter the expression of enzyme and transport genes regulated by FXR and PXR were studied in human intestinal cells. *In vitro* studies suggest a prominent role for progesterone-mediated interference with PXR signaling in the intestine during pregnancy that could contribute to altered xenobiotic transport. Further assessment of the ability of activated Fxr to modulate

hepatic and intestinal regulation of bile acid synthesis and transport pathways during pregnancy was investigated in mice. Treatment of pregnant mice with the specific Fxr agonist GW4064 restored the expression of bile acid synthesis enzymes and transporters towards levels typically observed in virgin mice. This study provides the molecular basis for a novel approach to restore bile acid homeostasis in patients with maternal cholestasis. Finally, loss-of-function studies in pregnant Fxr-null mice revealed that deficiency in the *Fxr* gene prevents pregnancy-mediated repression of key bile acid regulatory factors and transporters, largely in the intestines. Taken together, these data advance our understanding of alterations in chemical disposition during pregnancy, and the overall “procholestatic” state of pregnancy that sensitizes women to acquiring liver disease.

DEDICATION

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CHAPTER 1.1

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APPENDIX 1

Rudraiah S, **Moscovitz J**, Donepudi A, Campion S, Slitt A, Aleksunes L, Manautou J (2014) Differential Fmo3 gene expression in various liver injury models involving hepatic oxidative stress in mice. *Toxicology.* 325: 85-95. PMCID: PMC4428328

APPENDIX 2

Moscovitz J, Nahar S, Shalat S, Slitt A, Dolinoy D, Aleksunes L (2016) Correlation between conjugated bisphenol A concentrations and efflux transporter expression in human fetal livers. *Drug Metabolism and Disposition*. 44:1061-5. Invited short communication. PMID: 26851240

APPENDIX 3

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: GENERAL INTRODUCTION

1.1 Intestine-Liver Axis Physiology

The structure and function of the intestine and liver, as well as their interaction in maintaining digestion and nutrient homeostasis are well defined in the literature. The following information has been adapted from Berne & Levy Physiology (Koeppen and Stanton, 2010), unless otherwise indicated.

1.1.1 Small Intestine Physiology

Cell types

The small intestine, further defined as the duodenum, jejunum and ileum, is part of the greater gastrointestinal tract. The epithelium of the small intestine is composed of several important cell types that come in contact with the contents of the intestinal lumen, and is the focus of this section. Subsequent layers of the intestine are composed of connective tissue, muscle, and glands that secrete important endocrine and neurocrine factors to regulate digestion, absorption and excretion. The basic unit of the intestinal epithelium is the enterocyte. Enterocytes are columnar cells and critical to luminal digestion and absorption. Intermingled with enterocytes are goblet cells that secrete protective mucus to line the epithelium. Finally, most mamalliian species also have paneth cells that secrete a variety of exocrine proteins.

Structure

The entire gastrointestinal tract is organized into layers including: mucosa, submucosa, muscularis propria and adventitia (Fig. 1.1). To aid in its primary function of absorption, the shape and folds of the small intestine increase its surface area. The epithelium forms characteristic finger-like projections into the intestinal lumen called villi, whereas the bases of these folds are referred to as crypts (Fig. 1.1). Being that the epithelial cells of

the intestine have an advanced turnover and are highly proliferative, intestinal stem cells within crypts rapidly divide, mature, and progress up villi. Capillaries run below enterocytes of the villi to transport products of macronutrient digestion to the portal vein.

Functions of the Small Intestine

The small intestine is responsible for the completion of digestive processes and absorption of basic units of macronutrients including carbohydrates, proteins and lipids. Release of gastrointestinal mediators helps regulate the mixing and selective uptake of nutrients, ions and water, in addition to signaling to other organs to modify digestion. The osmotic gradient of the small intestine promotes the movement of water through tight junctions preventing significant loss. The first portion of the small intestine, the duodenum, neutralizes the pH of partially digested food emptying from the stomach in the form of acidic chyme. In addition to the alkaline mucus secreted by Brunner's glands, the duodenum releases secretin and cholecystokinin to promote the bicarbonate ion-rich secretions from the pancreas, and bile secretions from the gallbladder.

Carbohydrate digestion and uptake. Carbohydrates that will be absorbed by the small intestine are hydrolyzed to monosaccharides on the surface of intestinal epithelial cells in a process of brush border digestion. While many enzymes in the small intestine are membrane-bound, the proximity to the cell and uptake protects sugars from being modified by intestinal bacteria. The monomers of carbohydrates (i.e., sugars) are then absorbed into the enterocytes by different transporters, including the sodium/glucose symporter and members of the glucose transporter family.

Protein digestion and uptake. Proteins are partially digested prior to reaching the small intestine. Particularly in the duodenum, enterokinase activates the proteases released

from the pancreas to complete protein digestion to single amino acids. However, both single amino acids and small peptides can be absorbed in the small intestine with the assistance of peptide transporters. Short peptides may be broken down into single amino acids once in the enterocyte and prior to export.

Lipid solubilization and uptake. Though lipid processing begins in the stomach, it is the most complex of the macronutrient digestions that occur in the small intestine, requiring multiple steps. First, the intestinal muscular movement churns luminal contents for the emulsification of lipids, and greater access by lipolytic enzymes. As end products of lipolysis are formed from the initial lipid droplet, they are drawn into amphipathic mixed micelles with bile acids. Fatty acids can then cross the cell membrane due to their lipophilic nature, or enter the enterocyte similar to other products, via uptake transporters. The ATP-binding cassette (ABC) G5 and G8 transporters are responsible for cholesterol uptake. Upon entry into the enterocyte, lipids must be resynthesized to triglycerides, phospholipids and cholesterol esters, and form a chylomicron in complex with apolipoproteins. Lipids are released from the enterocyte via exocytosis and thereby evade portal vein passage to the liver.

Protective barrier. In addition to nutrients, orally ingested xenobiotic compounds are predominantly absorbed in the small intestine. This can include chemical contaminants associated with food and food storage, as well as drugs. Several ABC transporters with a wide variety of substrates are localized to the luminal membrane of the enterocyte and actively efflux exogenous compounds back into the intestinal lumen for fecal excretion (Fig. 1.2). Importantly, this includes the Multidrug Resistance Transporter, MDR1/*ABCB1*, Multidrug Resistance-Associated Protein, MRP2/*ABCC2*, and Breast Cancer Resistance Protein, BCRP/*ABCG2*.

1.1.2 Liver Physiology

Cell Types

The liver is a dynamic organ with multiple cell types that allow it to accomplish its main functions of energy homeostasis, detoxification and excretion. The parenchymal unit of the liver is the hepatocyte. In addition, the liver is composed of resident macrophage cells known as Kupffer cells, stellate cells and endothelial cells (Fig. 1.3). While many synthesis and metabolic functions proceed in the hepatocyte, Kupffer cells are phagocytic in nature and are responsible for particulate filtration. Stellate cells serve a supportive function, releasing critical hepatic growth factors and storing retinoid. The endothelial cells of the liver sinusoids have unique fenestrations and lack a basement membrane to make possible the diffusion of large molecules and proteins synthesized in the liver.

Structure

The liver is a multi-lobular organ. Hepatocytes are organized into plates, or chords, with blood and bile flowing in opposing directions through sinusoids and canaliculi, respectively (Fig.1.3). Adjacent hepatocytes form tight junctions, as well as canaliculi. The liver is highly perfused and hepatocytes are exposed to flowing blood from which many endo- and xenobiotics are filtered out for modification. Kupffer cells reside in the sinusoidal lumens for optimal filtration of bacteria arising from the intestine, such as endotoxin, and other particulates. Blood flows from the portal vein, and to a lesser extent, the hepatic artery, of the portal triad toward the central vein. Alternatively, bile is exported from hepatocytes into bile canaliculi, flowing toward the bile ducts of the portal triad. The liver lobules are further organized into zones with subtle nuances in function.

Hepatocytes of zone 1, closest to the portal triad, are relied upon for detoxification because they receive the greatest levels of oxygenation and nutrients from blood. Hepatocytes closer to the central vein in zone 3 are thought to have greater cytochrome P450 (CYP) enzyme content and bile acid synthesis activity.

Functions of the Liver

Energy homeostasis and protein synthesis. The liver is a major site of energy homeostasis, as many nutrients important to bodily function are produced, metabolized or stored there. Specifically, hepatocytes synthesize endogenous molecules such as coagulants, albumin, fatty acids, cholesterol and lipoproteins, and store cholesterol, in addition to glucose in the form of glycogen. The important processes of gluconeogenesis, glycogenesis and glycogenolysis predominantly occur in the liver.

Metabolism. There is a prominent role for hepatocytes in the metabolism of endogenous and xenobiotic compounds. In general, metabolic modifications made in the liver contribute to increased solubility and decreased toxicity for excretion. Reactions are carried out by both phase I and phase II enzymes. Important to phase I reactions are cytochrome P450 (CYP) enzymes, which oxidize a variety of exogenous and endogenous substrates. They are highly expressed in the liver, as well as the lungs and kidneys. The mouse liver expresses 31 of the 102 total Cyp enzyme isoforms that have been identified (Choudhary et al., 2003; Nelson et al., 2004). This is the largest number of Cyp isoforms present in a single mouse tissue. In contrast, 57 CYP genes and variants have been identified in the human genome (Nelson et al., 2004). While not all mouse Cyp isoforms have been shown to metabolize specific substrates, more than half exhibit sequence homology to human isoforms (Nelson et al., 2004; Hart et al., 2009). Phase II sulfotransferase enzymes are responsible for the metabolism of hormones and

steroids. Glutathione *S*-transferase enzymes catalyze the addition of the tripeptide glutathione (glycine-cysteine-glutamate) to substrates. Glutathione is an important defense in the cell for neutralizing electrophiles, protecting the cell from harmful reactive oxygen species, such as free radicals and organic hydroperoxides, and in the body for drug detoxification. Lastly, UDP-glucuronosyltransferase enzymes account for nearly half of total hepatic conjugation reactions. They catalyze the addition of glucuronic acid to a wide variety of small hydrophobic molecules.

Bile Formation. Cholesterol can be enzymatically converted into bile acids. Bile acids are present in significant quantities in bile, the excretory solution of the liver. In addition to bile acids, waste and biproducts, mixed micelles of bile are composed of phosphatidylcholine and cholesterol. After formation in the liver, the fate of bile is as an endogenous detergent to solubilize dietary fats and lipid-soluble vitamins in the intestinal lumen.

1.1.3 Intestine-Liver Interactions

Rather than returning to the heart for oxygenation, the venous blood supply leaving the intestine first goes to the liver. This creates a dynamic relationship between these organs that has implications for both endobiotic and xenobiotic handling.

Presystemic elimination. Orally ingested drugs are subject to presystemic elimination, or first pass metabolism, because they are absorbed in the intestine and further processed in the liver prior to becoming systemically available. Both metabolism and excretion can occur in enterocytes and hepatocytes, thereby reducing the bioavailability of an orally administered drug. Therefore, drugs that would be extensively metabolized in the liver

and rendered ineffective, due to their chemical and structural properties, must be delivered by different methods.

Enterohepatic recirculation. Bile acids are required in large quantities and are highly conserved through enterohepatic recirculation. Though bile acids are released into the duodenum, they are reabsorbed in the distal part of the small intestine, the ileum. In this way, 95% of bile acids are retained and returned to the liver via portal circulation while only 5% are excreted in feces daily. In humans, this equates to the retention of 15-30 g/day (20 mg/day for mice) of bile acids, and the excretion of only 0.5 g/day (Grundy et al., 1965). Ingestion of a meal stimulates the release of cholecystokinin from the duodenum, contraction of the gallbladder and release of bile into the intestinal lumen. The bile acids that are excreted from the liver into bile are predominantly conjugated to taurine, and to a lesser extent, glycine, and act as a detergent to optimize lipid absorption in the aqueous environment of the lumen. The balance between the hydrophobic and hydrophilic properties of bile acids corresponds to their ability to perform this function (Armstrong and Carey, 1982; Heuman, 1989; Heuman et al., 1989). Greater hydrophilicity equates to enhanced efficiency in emulsifying fats in the intestinal lumen. Taurine conjugated bile acids are more hydrophilic than glycine conjugated and unconjugated species (Martin et al., 2007). In contrast, reverse phase high performance liquid chromatography indicates the secondary bile acid lithocholic acid (LCA) is the most hydrophobic followed by deoxycholic acid (DCA), chenodeoxycholic acid (CDCA) and cholic acid (CA) (Heuman, 1989; Martin et al., 2007). Ursodeoxycholic acid (UDCA) is the most hydrophilic bile acid, and is therefore discussed in detail in Section 1.5 as a therapy for modifying bile acid pool composition in liver disease. The majority of bile acids that reach the intestine are deconjugated in the ileum by gut microbiota prior to

release into portal circulation. Extent and efficiency of deconjugation may vary by the species of bacteria and the bile salt hydrolase activity of each microbe.

1.2 Pregnant Physiology and Disposition

The growth of the placenta and fetus during pregnancy is accommodated by a number of physiological changes. Small cohort studies across gestation have predominantly contributed to our knowledge of pregnancy-related adaptations, as access to pregnant women and samples from patients are often limited. The culmination or peak of many adaptations occurs in the third trimester, and specifically at term.

Cardiovascular and hemodynamic adaptations. Multiple pulmonary system changes arise during pregnancy, including a reduction in total pulmonary resistance and a progressive increase in airway conductance (Gee et al., 1967). A recent meta-analyses was conducted of 39 studies quantifying cardiac output by echocardiography, impedance cardiography or inert gas rebreathing (Meah et al., 2016). The analysis confirmed that cardiac output escalates non-linearly throughout pregnancy, peaks early in the third trimester at a 30% increase compared to non-pregnant values, and subsequently declines until parturition (Meah et al., 2016). Though some studies have indicated enhanced heart rate contributes to this metric, heart rates peak at term (Meah et al., 2016). Other data suggest the greater cardiac output is more likely due to increased stroke volume (Chapman et al., 1998). During pregnancy, there is an average escalation in plasma volume by 1250 mL (30-50%) at 34-36 weeks of gestation (Hytten and Paintin, 1963; Pirani et al., 1973). Fetal size and parity can contribute to variations in plasma volume expansion (Rovinsky and Jaffin, 1965; Hytten, 1985).

Metabolic adaptations. Most metabolic organs are impacted by hemodynamic and hormonal changes observed in pregnancy. A study including 13 pregnant women revealed increased renal plasma flow as early as week 6 of gestation and persistent to week 36, which was indicated by para-aminohippurate clearance (Chapman et al., 1998). Pregnant Munich Wistar rats on gestation day 9 and pseudopregnant rats on day 9 also exhibit an up-regulation of renal plasma flow rate compared to virgin controls (Baylis, 1982). In combination with the expanded plasma volume, there is a net effect of increased glomerular filtration rate. Enhanced glomerular filtration rate has been quantified directly by inulin clearance in pregnant women compared to their own pre-conception values (Chapman et al., 1998). In a study conducted in 67 pregnant subjects between 10 and 40 weeks of gestation, and 22 non-pregnant subjects, investigators reported a 50-60% increase in hepatic perfusion rate during the third trimester of pregnancy (Nakai et al., 2002). The elevation in hepatic perfusion was attributed to increased portal vein cross-sectional area and blood flow, while no significant changes were observed in the hepatic artery (Nakai et al., 2002). Finally, pregnant women experience decreased gastrointestinal motility which is often evidenced by constipation (Parry et al., 1970). This same phenomenon has been demonstrated in pregnant mice from gestation days 11 to 19 (Datta et al., 1974), as well as modeled with high dose progesterone (5 mg/kg) treatment in male rats (Liu et al., 2002). Likewise, progesterone has been implicated in the increased gastrointestinal residence time for pregnant humans (Wald et al., 1981; Wald et al., 1982).

1.2.1 Hormones in Pregnancy

Serum hormone profiles. As alluded to previously, pregnant women experience vast changes to steroid hormone profiles, and the introduction of placental hormones to

circulation. By term, maternal circulating concentrations of progesterone reach 130 to 150 ng/mL (Creasy and Resnik, 2009). Circulating estradiol levels in late pregnancy increase by the same magnitude as progesterone (100-fold), to approximately 40 ng/mL (Creasy and Resnik, 2009). Estriol, an estrogen synthesized by the placenta from fetal androgen precursors, also reaches significant production in the third trimester, with maternal serum levels of 12 to 20 ng/mL (Creasy and Resnik, 2009). While in non-pregnant women the adrenal glands and ovaries are responsible for androgen production, during pregnancy synthesis occurring in the feto-placental unit leads to increases in testosterone and dehydroepiandrosterone (DHEA) in maternal blood. In the third trimester of pregnancy, maternal circulating concentrations of testosterone reach 13.3 pmol/L; double the levels of non-pregnant women (Wilke and Utley, 1987). DHEA levels range widely, and reach up to 50 nmol/L in the third trimester, as compared to non-pregnant women in which they may reach up to 40 nmol/L (Tagawa et al., 2004). Cortisol concentrations are induced by 3-fold, and range from 0.5 to 0.8 μM in late pregnancy (Soldin et al., 2005). As would be expected, steroid hormone metabolites are also elevated during pregnancy. Pregnant women in the third trimester exhibit 80-fold higher levels of the progesterone metabolites allopregnanolone-sulfate (PM4S) and epiallopregnanolone-sulfate (PM5S) (Abu-Hayyeh et al., 2010). In addition to steroid hormones and their metabolites, the placenta produces human chorionic somatomammotropin, or placental lactogen, which becomes the most abundant hormone in late pregnancy, peaking at 5 to 15 μg/mL prior to parturition (Frankenne et al., 1988). Sex hormones can be bound in circulation by sex hormone binding globulin, albeit at different affinities. Levels of sex hormone binding globulin increase by nearly 6-fold in the maternal circulation during the third trimester of pregnancy, in association with increased total sex hormone levels (Wilke and Utley, 1987).

Though placentation differs between humans and mice, similar trends are observed to hormone levels in late gestation. Pregnant mice exhibit a 4- to 10-fold increase in progesterone levels by late gestation (Piekorz et al., 2005; Aleksunes et al., 2012). Importantly however, plasma progesterone concentrations peak at gestation days 14-17, and already begin to decline prior to parturition (McCormack and Greenwald, 1974). Estradiol levels double or triple in mice by gestation day 14 and remain high (McCormack and Greenwald, 1974; Aleksunes et al., 2012). The mouse placenta produces two varieties of placental lactogen, placental lactogen-I which peaks from gestation days 8 to 11, and placental lactogen-II which is not synthesized until gestation day 9 (Soares et al., 1982; Ogren et al., 1989).

Steroid hormone signaling. Free or unbound fractions of sex hormones in maternal circulation are available to cross plasma membranes and act on their cognate endocrine nuclear receptors to transduce signaling pathways and alter molecular processes. Endocrine nuclear receptor signaling of steroid hormones results in transcriptional regulation of target genes (genomic regulation), though steroid hormones regulate cellular function through autocrine, paracrine, and nongenomic signaling as well. Estrogens, progestins, androgens, and glucocorticoids bind the classical estrogen receptor (α/β), progesterone receptor, androgen receptor, and glucocorticoid receptor, respectively. Ligand binding to a cytoplasmic endocrine nuclear receptor results in receptor translocation to the nucleus, homodimerization, binding to hormone response elements in the promoter region of target genes and ultimate alteration of transcription. Endocrine nuclear receptors are in complex in the cytoplasm with chaperone proteins, such as heat shock proteins, which keep them active but prevent signaling in the absence of ligand binding. In addition to classical endocrine receptors, steroid hormones can bind and activate orphan nuclear receptors. For example, an orphan nuclear

receptor was identified based on its ability to be bound and activated by pregnanes, including progestins (Kliewer et al., 1998). For this reason, the pregnane X receptor (PXR) was originally termed the steroid and xenobiotic receptor (Blumberg et al., 1998). PXR can also be activated by glucocorticoids such as dexamethasone (Kliewer et al., 1998). Through orphan nuclear receptor binding, steroid hormones can interact with several different receptors, with overlap in activity.

Placental lactogen signaling. Unlike steroid hormones, placental lactogen belongs to the growth hormone family (in humans) or prolactin family (in rodents). In humans, placental lactogen therefore is preferentially a ligand of the membrane-bound prolactin receptor, though it also has activity towards the growth hormone receptor (reviewed in Soares, 2004). Binding of placental lactogen to either receptor can initiate several different types of signaling, including janus kinase/ signal transducers and activators of transcription (Stat) proteins (Takeda et al., 1997) and phosphoinositide 3-kinase (Lee et al., 2014) pathways.

1.3 Bile Acid Homeostasis in Pregnancy

1.3.1 Serum Bile Acid Profiles

Conflicting reports have been published regarding serum bile acid levels among non-pregnant and pregnant women. In one of the larger studies (n=44-49 subjects per gestational week), serum bile acid levels were generally consistent, albeit falling within a broad range of 0.3 to 9.8 µmol/L, in healthy women at gestation weeks 12, 20, 28 and 36 (Egan et al., 2012). Several smaller studies have quantified individual bile acids in uncomplicated pregnancies as control groups. Castano et al. (2006), observed increased total bile acids, DCA and UDCA levels in healthy pregnancies (n=18) in

comparison to levels in non-pregnant women (n=10) (Castano et al., 2006). A subsequent study demonstrated that women with uncomplicated pregnancies exhibited the highest levels of CDCA (0.32 µmol/L), followed by CA (0.25 µmol/L) and DCA (0.27 µmol/L), though no comparison was made to non-pregnant subjects (Geenes et al., 2014b). Variability in control groups, sampling time points, and sample size has made comparison of serum bile acid levels across studies difficult.

1.3.2 Bile Acid Synthesis

CYPs synthesize bile acids in the liver via the classic or alternative pathway (Fig. 1.4). The primary bile acid formed by the classic pathway is CA. While cholesterol-7α-hydroxylase, or CYP7A1, is the rate-limiting enzyme in this pathway, sterol 12α-hydroxylase, or CYP8B1 is also critical. The primary bile acid formed from the alternative pathway is CDCA in humans, and murcholic acid (MCA) in mice. CYP27A1, 7B1 and 39A1 are important to the alternative pathway, though Cyp27a1 is not established as rate limiting in mice.

Pregnant mice exhibit enhanced expression of classic pathway enzymes Cyp7a1 and 8b1 by up to 6- and 2-fold, respectively, while alternative pathway enzymes Cyp27a1 and 39a1 are repressed up to 50% (Milona et al., 2010a; Aleksunes et al., 2012). Cyp7a1, 8b1, 27a1 and 7b1 expression in Sprague Dawley rats has been determined throughout gestation. Interestingly, authors noted a reduction in Cyp8b1 mRNA levels, while expression of other bile acid synthesizing enzymes remained constant (Zhu et al., 2013).

1.3.3 Bile Acid Conjugation

Bile acids are conjugated to the amino acids taurine or glycine prior to being excreted across bile canaliculi. As is similar with many phase II reactions, conjugation makes bile acids less toxic and increases their ability to promote the absorption of lipids and lipid soluble vitamins in the intestine. Two critical hepatic conjugating enzymes include bile acid CoA ligase (Bal) and bile acid CoA:amino acid N-acetyltransferase (Baat) (Schersten et al., 1967; Killenberg, 1978; Kimura et al., 1983; Johnson et al., 1991; Falany et al., 1994). Similar to Cyps in the alternative pathway, both Bal and Baat mRNA are down-regulated 50-75% on gestation day 14, while Bal mRNA expression remains low on gestation day 17 (Aleksunes and Klaassen, 2012). Likewise, Baat protein levels were reduced in pregnant mice on gestation day 17 (Aleksunes et al., 2012). A reduction in conjugation coupled with changes observed in bile acid synthesis may contribute to a net increase in the toxicity of the bile acid pool during pregnancy.

Bacteria in the intestine can produce secondary and tertiary bile acids, as well as perform deconjugation prior to recirculation. CA can be dehydroxylated to DCA, CDCA to LCA, and α/β-MCA to UDCA or ω-MCA (Martin et al., 2007). Deconjugated bile acids are then free to recirculate back to the liver. It is understood that differences in bacterial species of the microbiome contribute to differing bile acid pool compositions (Martin et al., 2007). Moreover, there are drastic changes to the gut microbiome in pregnancy, which varies across trimesters (Koren et al., 2012). However, little has been reported in reference to the implications of pregnancy-related microbiome changes to bile acid handling.

1.3.4 Bile Acid Transport

Once excreted into the intestinal lumen, bile acids can enter the enterocyte via the apical sodium dependent bile acid transporter (Asbt, *Slc10a2*) (Fig. 1.2) (reviewed in Dawson, 2011). The organic solute transporter Ostα/β/*Slc51a/b* heterodimer and Mrp3/*Abcc3* transporter extrude bile acids into portal circulation. Portal bile acids are taken up into hepatocytes predominantly by the sinusoidal transporter, Ntcp/*Slc10a1* (Fig. 1.5). Mdr transporters are responsible for the canalicular secretion of amphipathic molecules and cations (Mdr1a/1b/*Abcb1a*/*1b* isoforms in rodents and MDR1 in humans, also known as P-glycoprotein) and phospholipids (Mdr2/*Abcb2* isoform in rodents and MDR3 in humans). The excretion of organic anions from the liver is primarily performed by Mrp transporters. Hepatic Mrps are located on both the sinusoidal (Mrp/*Abcc* 1, 3, 4, 6) and canalicular (Mrp2/*Abcc2*) plasma membrane (reviewed in Klaassen and Aleksunes, 2010). The canalicular efflux transporters, bile salt export pump (Bsep/*Abcb11*) and Mrp2 (Keppler et al., 1997; Stieger et al., 2007) are primarily responsible for removing bile acids from the liver. Similar to their function in the intestines, the sinusoidal efflux transporters, Ostα/β and Mrp3 pump small amounts of bile acids back into the blood circulation.

The literature shows that in mice, maternal hepatic Ntcp and Bsep mRNA, protein, and localization to their respective membranes are significantly reduced on gestation days 14, 17, and 18 (Milona et al., 2010a; Aleksunes et al., 2012). Mrp2 and Mrp3 mRNAs are similarly down-regulated during the same gestational time points in mice, with Mrp3 repressed by nearly 80% and Mrp2 by 40% (Milona et al., 2010a; Aleksunes et al., 2012). Expression data from rat livers confirms similar repression of Ntcp and Mrp3, but not Bsep (Zhu et al., 2013). Sex hormones have been demonstrated to differentially regulate transporters.

Estrogen. The regulation of Ntcp mRNA expression by 17β-estradiol has been studied in gonadectomized rodents. Ovariectomized mice had an approximate 50% reduction in mRNA expression of Ntcp compared to intact females (Cheng et al., 2007). Ovariectomized mice with a 21-day subcutaneous implant containing 0.5 mg 17β-estradiol did not exhibit the same attenuation in Ntcp mRNA expression (Cheng et al., 2007). Alternatively, ovariectomized Sprague Dawley rats exhibited a 2.5-fold enhancement of Ntcp mRNA expression (Simon et al., 2004). Administration of 100 µg/kg/day of 17β-estradiol of ovariectomized rats reduced Ntcp expression near intact female control levels (Simon et al., 2004). Expectedly, this divergent role of estrogen in Ntcp regulation in rodents is also exemplified by higher Ntcp expression in female mouse livers as compared to male mouse livers, and male rat livers as opposed to female rat livers.

Due to the significant role of Bsep in the recirculation of bile acids, mechanisms of repression during mouse pregnancy have been explored in detail, and point to a prominent role of Estrogen receptor alpha (Erα) and Farnesoid X receptor (Fxr). *In vivo*, live imaging of mice with an injected luciferase Bsep promoter reporter confirmed decreased Bsep transcription in late pregnancy, which inversely correlated with serum 17β-estradiol levels (Song et al., 2014). Furthermore, Bsep down-regulation during late gestation was attenuated in Erα-null mice treated subcutaneously with 5 mg/kg 17β-estradiol daily for 5 days. The authors propose indirect transcriptional repression of the Bsep gene through the protein-protein interaction of Erα and Fxr, supported by the coimmunopreciptation of such complexes *in vitro* and *in vivo* (Song et al., 2014). In a subsequent study, 17β-estradiol treatment (100 nM) decreased the Fxr-mediated recruitment of coactivator proliferator-activated receptor gamma coactivator-1, and enhance nuclear receptor corepressor recruitment to the human *BSEP* promoter in Huh7

hepatoma cells (Chen et al., 2015). In contrast, male rats receiving a single (noncholestatic) dose of ethinylestradiol exhibited a 4-fold induction of Mrp3 mRNA expression (Ruiz et al., 2013). Estrogen-enhanced Mrp3 mRNA expression was recapitulated in primary rat hepatocytes treated with 10 µM ethinylestradiol, and was attenuated by the addition of the Er inhibitor, ICI182/780 (Ruiz et al., 2013).

Progesterone. The progesterone metabolites, PM4S and PM5S, have been shown to interfere with NTCP uptake *in vitro* (Abu-Hayyeh et al., 2010). Investigators found that both progesterone metabolites were competitive inhibitors of Na^+-dependent and Na^+-independent [^{3}H] taurocholic acid uptake in primary human hepatocytes and NTCP-expressing *Xenopus laevis* oocytes. Importantly, IC_{50} and K_i values for NTCP inhibition were equivalent to concentrations observed in women during the third trimester of pregnancy (~5-7 µM) (Abu-Hayyeh et al., 2010). A reduction in activity of Bsep in the presence of progesterone and its metabolites has also been demonstrated in *Xenopus laevis* oocytes transfected with rat Bsep (Vallejo et al., 2006). Progesterone, PM4S and PM5S inhibited Bsep mediated transport of radiolabeled cholic acid methyl ester by 20-50% (Vallejo et al., 2006).

Additional hormone interactions. A once daily 50 mg/kg injection of wild-type mice with the glucocorticoid dexamethasone for 4 days increased mRNA expression of Ntcp (Cheng et al., 2007). This induction was largely attenuated in Pxr-null mice, demonstrating that Pxr was necessary, in part, for the induction of Ntcp mRNA (Cheng et al., 2007). Mouse placental lactogen-I (0.5 µg/mL) induced Ntcp mRNA expression by 5-fold in primary rat hepatocytes (Cao et al., 2001). Both reports identify Stat response elements in the Ntcp promoter region, and implicate Stat5 activation in its transcriptional regulation (Cao et al., 2001; Cheng et al., 2007). Sex hormones may potentiate or

negate each other's ability to regulate transporter expression in a state such as pregnancy. For instance, ovariectomized rats treated with ovine prolactin for 7 days exhibited a 7-fold induction of Ntcp mRNA (Cao et al., 2004). This up-regulation was attenuated by co-treatment with 17β-estradiol, as was prolactin-mediated Stat5a transactivation in cells co-transfected with Erα (Cao et al., 2004).

1.4 Enterohepatic Nuclear Receptor Signaling in Pregnancy

In a normal physiological state, bile acid synthesis, conjugation and recirculation are tightly regulated by multiple transcription factors. These same regulatory pathways also control enzymes and transporters that determine xenobiotic disposition. Transcription factors in the nuclear receptor family are of particular importance, and several have been demonstrated to be suppressed in the maternal mouse liver, including Fxr, Pxr, constitutive androstane receptor (Car), liver X receptor (Lxr) and peroxisome-proliferative associated receptor alpha (Pparα) (Sweeney et al., 2006; Koh et al., 2011; Aleksunes et al., 2012; Wen et al., 2013). Mouse Erα mRNA is induced, providing a mechanism for increased 17β-estradiol levels during pregnancy to influence cholesterol and lipid pathways (Wen et al., 2013). Erα/β mRNA and protein are also enhanced in pregnant rats on gestation day 20-21 (Cao et al., 2004). In addition to species differences in Ntcp transporter regulation (Section 1.3.4), contrasting data has been observed in rodents regarding nuclear receptor expression during pregnancy. While there was no change in rat Fxr mRNA, there was an induction of Fxr protein on gestation day 14 (Zhu et al., 2013). Additionally, rat Pparα gene expression was induced on gestation day 14, while Erα mRNA was repressed on gestation days 10 and 14 (Zhu et al., 2013). Zhu et al. (2013) propose that in Sprague Dawley rats, unlike mouse models, bile acid homeostasis is sustained during pregnancy through unaltered Fxr regulation.

Fxr is highly expressed in the ileum of the small intestine and liver, further implicating it in multiple roles of the intestine-liver axis. Most importantly in the small intestine, Fxr mediates the induction of human fibroblast growth factor FGF19/mouse Fgf15, which binds to its hepatic receptor Fgfr4 and suppresses bile acid synthesis genes Cyp7a1 and 8b1 in the liver (Inagaki et al., 2005; Yu et al., 2005). In response to bile acid binding in the hepatocyte, Fxr up-regulates the transcription of small heterodimer (Shp), which works with Fgf15 to suppress the expression of Cyp7a1. The human liver can secrete FGF19 under cholestatic conditions, whereas this same adaptation has not been observed in the mouse liver (Schaap et al., 2009; Song et al., 2009). Fxr can directly transactivate hepatic bile acid transporters Ntcp, BSEP/Bsep, MDR3 and Ostβ (Lu et al., 2000; Ananthanarayanan et al., 2001; Denson et al., 2001; Abu-Hayyeh et al., 2010; Thomas et al., 2010; Huang et al., 2011). Of the nuclear receptors mentioned previously, Fxr and its repression in mice is the most highly studied in relation to maternal bile acid homeostasis.

During mouse pregnancy, elevated bile acids have been attributed to impaired Fxr function by increased steroid hormone levels (Abu-Hayyeh et al., 2010; Milona et al., 2010b; Aleksunes et al., 2012; Abu-Hayyeh et al., 2013a). Unpublished data from our laboratory demonstrates that FGF19 levels in the serum of non-fasted healthy pregnant subjects (n=40 per group) decrease 15% from the second to third trimester. Whether total bile acids in humans vary too greatly to observe alterations in small cohorts or remain unchanged throughout gestation (discussed in Section 1.3.1), media supplemented with serum from healthy pregnant women resulted in a down-regulation of Shp mRNA expression in rat Fao hepatoma cells (Milona et al., 2010a). Bile acid synthesis is governed by distinct mechanisms; negative feedback from Fxr and Shp, as

well as positive feedback from Lxr. Lxr is a positive regulator of Cyp7a1 and is inhibited by an interaction with Shp (Brendel et al., 2002), allowing for negative feedback (from Fxr) to be dominant in maintaining homeostasis. Interestingly, the reduction of Shp during pregnancy does not correspond with an induction of Lxr, suggesting it may be repressed by other mechanisms.

In addition to Fxr, Pxr and Car are expressed in the ileum and help coordinately regulate bile acid disposition in this tissue (Modica et al., 2010). CAR/Car and PXR/Pxr activation have been demonstrated to induce the important bile acid transporters MRP/Mrp 2 and 3 in humans and rodents, as well as modify NTCP and BSEP transcription in humans (Kiuchi et al., 1998; Cherrington et al., 2002; Kast et al., 2002; Xiong et al., 2002; Teng et al., 2003; Maher et al., 2005; Jigorel et al., 2006; Petrick and Klaassen, 2007; Olinga et al., 2008; Aleksunes et al., 2009). In female rats, once daily injections with estradiol benzoate (1 mg/kg/day) for 5 days resulted in plasma concentrations of estradiol similar to those reached in the first trimester of pregnancy (Choi et al., 2013). This estradiol benzoate treatment regimen reduced Pxr expression by 70% in comparison to vehicle-treated female rats (Choi et al., 2013).The authors however went on to note dissimilar changes to Cyp gene expression between estradiol benzoate treated rats and pregnant humans.

1.5 Intrahepatic Cholestasis of Pregnancy

During the third trimester, women can begin to develop symptoms of cholestatic liver disease. In the United States, the incidence of intrahepatic cholestasis of pregnancy (ICP) varies geographically, and ranges from 0.32 to 5.6% of pregnancies (Laifer et al., 2001; Lee et al., 2006). Globally, the highest reported rates of ICP are found in Chile,

and may occur in as many as 15.6% of pregnancies (Reyes et al., 1978). ICP enhances the transfer of bile acids from mother to fetus, which increases the risk of fetal morbidity and mortality due to preterm labor, fetal cardiac distress, and intrauterine death (Papacleovoulou et al., 2013; Wikstrom Shemer et al., 2013; Geenes et al., 2014a). Pregnant women with ICP are at an increased risk of concomitant diseases including gestational diabetes and preeclampsia (Marschall et al., 2013; Wikstrom Shemer et al., 2013; Raz et al., 2015). Recent research in humans and mice suggests that women with ICP have greater susceptibility to develop hepatobiliary disease after delivery, while offspring born to mothers with ICP are at higher risk of developing acute respiratory disorders and metabolic diseases (Marschall et al., 2013; Papacleovoulou et al., 2013). Risk factors for ICP include hepatitis C virus, low selenium or vitamin D levels, multiple pregnancies and advanced age at the time of gestation (reviewed in Floreani and Gervasi, 2016). It has been determined that a subset of ICP cases can be attributed to genetic variants of important canalicular transporters and bile acid regulatory factors (Table 1.1).

BSEP/ABCB11. Using a candidate based approach, investigators screened ICP cohorts from both the United Kingdom and Europe (n=491) and over 200 control women for common mutant alleles of *ABCB11* (E297G and D482G), as well as a preliminarily identified mutation (N591S), and a variant associated with drug induced cholestasis (V444A) (Dixon et al., 2009). Data from this study suggest that the single nucleotide polymorphism V444A was the greatest risk factor in the studied ICP cohort, while other mutations rarely occurred (Dixon et al., 2009). A subsequent study conducted by the same group in a slightly larger cohort (n=563) identified 6 additional polymorphisms in *ABCB11* that were associated with ICP (Dixon et al., 2014).

MDR3/ABCB4. Several mutations and splicing variants have been identified in the *ABCB4* gene that encodes the canalicular phosphatidylcholine transporter, MDR3. A heterozygous single-nucleotide deletion (1712delT) was observed in a single family of which four women had previously demonstrated symptoms or negative clinical fetal outcomes associated with ICP (Jacquemin et al., 1999). A less common polymorphism corresponding to a mutant allele in exon 14 (A546D) of the *ABCB4* gene was subsequently discovered in an ICP patient with elevated serum γ-glutamyl transpeptidase, specifically (Dixon et al., 2000). Several follow up studies confirmed the existence of an *ABCB4* splicing variant (c.3486+5G>A) in an ICP patient (Schneider et al., 2007), non-synonymous heterozygous mutations in exons 14, 15 and 16 in a small cohort (Floreani et al., 2008) and 6 single-nucleotide polymorphisms around the *ABCB4* loci in a larger case control study (Dixon et al., 2014). There have been no studies conducted to characterize the frequency or associated risk of these variants with ICP at large.

FXR/NR1H4. An FXR sequencing study was conducted in 92 British ICP cases and followed by case-control studies in multiple ICP cohorts to determine allele frequencies for *NR1H4* gene variants (Van Mil et al., 2007). The results suggest a significant association of ICP with the functional variants M173T and -1g>t (Van Mil et al., 2007). This finding supports the hypothesis that further perturbation of the FXR pathway beyond normal alterations to human pregnant physiology may contribute to acquired liver disease.

Data demonstrate women with ICP exhibit increased serum levels of more hydrophobic bile acids, including LCA and free bile acids, as compared to healthy pregnant women (Castano et al., 2006). ICP patients also present with higher levels of certain sex

hormones. In subjects weeks 33-36 of gestation, the progesterone metabolite PM5S was increased 330% in the serum of ICP patients (n=15) as compared to healthy pregnant patients (n=12) (Abu-Hayyeh et al., 2013a). Earlier studies demonstrated raised levels of serum 3α-sulfated progesterone metabolites in 6 ICP cases compared to 3 normal pregnancies, though these data were not presented as absolute values (Meng et al., 1997). No direct comparison between ICP patients and healthy patients was made, as gestational week sampling was between 31-34 weeks and 36-38 weeks, respectively.

Several drugs, albeit with limited clinical benefit, have been prescribed to pregnant women to alleviate the symptoms of ICP including phenobarbital, hydroxyzine, cholestyramine and dexamethasone. The current drug to treat ICP is ursodeoxycholic acid (UDCA, Actigall®), which not only alleviates pruritus but also reduces serum total bile acid, alanine aminotransferase and bilirubin levels (Joutsiniemi et al., 2014). The mechanism of action for UDCA remains largely unknown and is efficacious for only a subset of pregnant women (Burrows et al., 2001). Studies performed on wedge liver biopsies from non-pregnant, healthy human patients showed induction of both hepatic BSEP and MDR3 mRNA and protein expression with UDCA treatment (Marschall et al., 2005), while other studies have shown that UDCA alters the bile acid pool composition, reducing toxic, hydrophobic bile acids (Paumgartner and Beuers, 2004). More recently, combination treatment with UDCA and rifampicin was demonstrated to be effective in a small cohort of women with severe ICP (total bile acids equal to or exceeding 100 μmol/L) (Geenes et al., 2015). Even with successful pharmacological control, obstetricians still recommend early delivery, by 37 weeks, as part of the management plan for ICP.

Current research is being directed at diagnostic and predictive markers for ICP. One study identified serum autotaxin was increased in women diagnosed with ICP in comparison to women with uncomplicated pregnancies, and when compared to women with other pregnancy-related disorders (Kremer et al., 2015). A subsequent study went further and analyzed serum samples for predictive markers of ICP prior to onset. The authors found that in the first trimester, and prior to the onset of symptoms of ICP, the levels of three sulfated progesterone metabolites were prognostic for ICP (Abu-Hayyeh et al., 2016). Both sulfated progesterone metabolite and autotaxin serum levels may assist in differentiating ICP from other pruritus-related diseases to determine pregnant patients at risk for negative outcomes versus benign cases.

1.6 Modulation of the Farnesoid X Receptor

1.6.1 Fxr Agonism

Several specific pharmacological Fxr agonists have been synthesized to study the role of Fxr in various cholesterol, glucose and bile acid-related diseases. This includes the compounds fexaramine, WAY-362450 and GW4064 (Fig. 1.6). Because FGF19 signaling is intimately related to intestinal FXR activation, exogenous FGF19 has been studied as a therapy for gastrointestinal diseases.

GW4064. GW4064 is a non-steroidal FXR agonist that was first identified in 2000 (Maloney et al., 2000). In cholestasis resulting from bile duct ligation (BDL, extrahepatic) or administration of the chemical hepatotoxicant alpha-naphthylisothiocyanate (ANIT, intrahepatic) in rats, GW4064 treatment improved serum biochemistries, and decreased necrosis and inflammatory response in bile ducts (Liu et al., 2003). Treatment of gallstone-susceptible C57BL/6 mice for 1 week with 100 mg/kg GW4064 resolved cholesterol crystallization in the gallbladder and reduced inflammation in the gallbladder

intestinal epithelium (Moschetta et al., 2004). In a study looking at models of diabetes and obesity, GW4064 was able to lower serum triglyceride levels (Watanabe et al., 2004). In wild-type mice, a mouse model of type 2 diabetes, and a mouse model of obesity, treatment with 30 mg/kg of GW4064 for 3 days decreased serum triglyceride levels, as well as improved insulin resistance in obese mice (10 days of GW4064) (Watanabe et al., 2004; Cariou et al., 2006). Further, the reduction of serum triglyceride levels in wild-type mice was abrogated in Shp-null animals, suggesting that Fxr signaling is necessary for the activity of GW4064 (Watanabe et al., 2004). Subsequent studies in mouse models of diabetes (*db/db* mice) revealed plasma glucose, triglyceride and cholesterol levels were all reduced by approximately 50% treated twice daily for 5 days with 30 mg/kg of GW4064 (Zhang et al., 2006).

Fexaramine. Oral administration of fexaramine to wild-type mice was confirmed to activate ileal Fxr independently of hepatic Fxr, and dose dependently reduce weight gain in mice fed a high fat diet for 14 weeks (Fang et al., 2015). Specifically, there was a reduction of overall fat mass and both types of adipose tissue in mice concurrently administered 50 mg/kg fexaramine while consuming the high fat diet. Importantly, ileal Fgf15 mRNA and protein expression was induced 3-fold. Further, obese mice had a 30% reduction in bile acid pool size and taurocholate conjugated bile acids (CA), and an increase in secondary bile acids (ωMCA, LCA) (Fang et al., 2015).

WAY-362450. A recent study proposed administration of wild-type mice with 5 mg/kg 17α-ethinylestradiol from gestation days 14 to 20 produces a model of maternal cholestasis (Wu et al., 2015). While serum, hepatic, and amniotic fluid total bile acids were elevated with 17α-ethinylestradiol treatment alone, this effect was attenuated in animals co-treated with 30 mg/kg of the Fxr agonist WAY-362450 (Wu et al., 2015).

Additionally, placentas collected from mice that were co-administered 17α-ethinylestradiol and WAY-362450 exhibited significantly less edema and trophoblast apoptosis, compared to animals treated with 17α-ethinylestradiol alone. Authors noted the reversal of pathological changes with co-administration was paralleled by the induction of the Bsep transporter in placentas (Wu et al., 2015).

Exogenous FGF19. Native FGF19 has been implicated in cell proliferation and tumorigenesis. Investigators demonstrated that transgenic mice overexpressing FGF19 cross-bred with FGFR4-null mice do not form liver tumors, while FGF19 transgenic mice that are FGFR4 positive develop hepatocellular carcinomas (French et al., 2012). Further, transgenic FGF19 mice, as well as wild-type mice injected with exogenous FGF19 exhibited evidence of increased gross tumor formation and incorporation of 5-bromo-2′-deoxyuridine into hepatocytes at 10 to 12 months, as compared to appropriate controls (Nicholes et al., 2002). Therefore, modifications to FGF19 have been made resulting in a nontumorigenic variant for consideration as an agonist of hepatic FXR (Zhou et al., 2014). The variant of FGF19, M70, has recently been synthesized and characterized (Luo et al., 2014). Similar to other studies, its efficacy in protecting mice from BDL and administration of the chemical ANIT was tested. Twice daily subcutaneous injections of M70 for 9 days reduced both bile acid synthesis and the excess production of hepatic and total bile acids in FVB mice compared to vehicle-injected controls (Luo et al., 2014). In addition, administration of M70 reduced hepatic necrosis observed in both models of cholestasis (Luo et al., 2014).

1.6.2 Fxr Deficiency

Fxr deficiency has been implicated in enhanced susceptibility to cholestasis, gallstone disease, nonalcoholic steatohepatitis, and spontaneous formation of liver and colon

tumors in mice (Kim et al., 2007b; Yang et al., 2007; Kong et al., 2009; Maran et al., 2009). Both male and female Fxr-null mice spontaneously develop various liver tumors including lesions, adenomas and carcinomas by 12 to 15 months of age (Kim et al., 2007b; Yang et al., 2007). Mice lacking Fxr also exhibit increased serum and liver total bile acid levels, pro-inflammatory markers (interleukins 1β and 6, interferon-γ, tumor necrosis factor-α), and hepatic inflammatory responses. An impaired response to exogenous cholic acid and enhanced liver damage suggest these mice are more susceptible to liver specific injury (Yang et al., 2007). Similar to the aforementioned studies regarding liver damage, sensitivity to tumorigenesis has been reported in the intestines of Fxr-null mice. Mice deficient in Fxr had greater colon cell proliferation and up-regulation of similar pro-inflammatory cytokines as observed in the liver (Maran et al., 2009). Fxr-null mice exhibited increased quantity and size of adenocarcinomas 6 months after a 6 week challenge with 10 mg/kg of the carcinogen, azoxymethane (Maran et al., 2009).

Fxr deficiency has not only been implicated in tumorigenesis, but also cholestatic disorders. Fxr-null mice, in combination with high fat diet or LDL-null background exhibited pathologies similar to non-alcoholic steatohepatitis (Kong et al., 2009). An enhanced pro-inflammatory cytokine profile, in addition to macrosteatosis and elevated serum ALT and ALP levels were noted in these mice (Kong et al., 2009). Fxr-null mice challenged with a one-week lithogenic diet exhibited more turbid gallbladder bile with increased cholesterol precipitation and crystallization in comparison to wild-type mice fed the same diet (Moschetta et al., 2004). Finally, Fxr-null mice exhibited a more hydrophobic bile acid pool, greater inflammation in the gallbladder, and increased plasma and liver cholesterol and triglyceride levels (Moschetta et al., 2004).

1.7 Research Objective and Hypothesis

Limited human studies are available to support the global down-regulation of hepatic conjugation and transport, as well as induction of classic bile acid synthesis that has been characterized in mice. Furthermore, complimentary studies in the intestine have not been conducted. Understanding the role(s) of human/rodent enterohepatic nuclear receptor signaling in the regulation of xenobiotics and bile acids during pregnancy will aid in understanding appropriate modifications to dosing regimens and designing future treatment strategies that specifically target the underlying mechanism of action of ICP. The central hypothesis is that high concentrations of circulating hormones impair nuclear receptor signaling in the intestine-liver axis leading to altered xenobiotic and endobiotic disposition and heightened susceptibility to liver disease during pregnancy. Four aims have been designed to test this hypothesis:

- Aim 1. Assess time-dependent changes in the mRNA and protein expression patterns of intestinal bile acid homeostasis and transport pathways in pregnant mice.
- Aim 2. Determine whether steroid and placental hormones alter bile acid signaling and transport in the intestines.
- Aim 3. Determine the ability of Fxr activation to restore bile acid synthesis and transport expression in pregnant mice.
- Aim 4. Assess the impact of loss of hepatic and intestinal Fxr signaling on pregnancy-induced bile acid changes.

The studies completed in this thesis will help to understand the mechanisms underlying altered enterohepatic nuclear receptor signaling during pregnancy, and point to a potential therapeutic target for treating pregnant women with cholestatic liver disease.

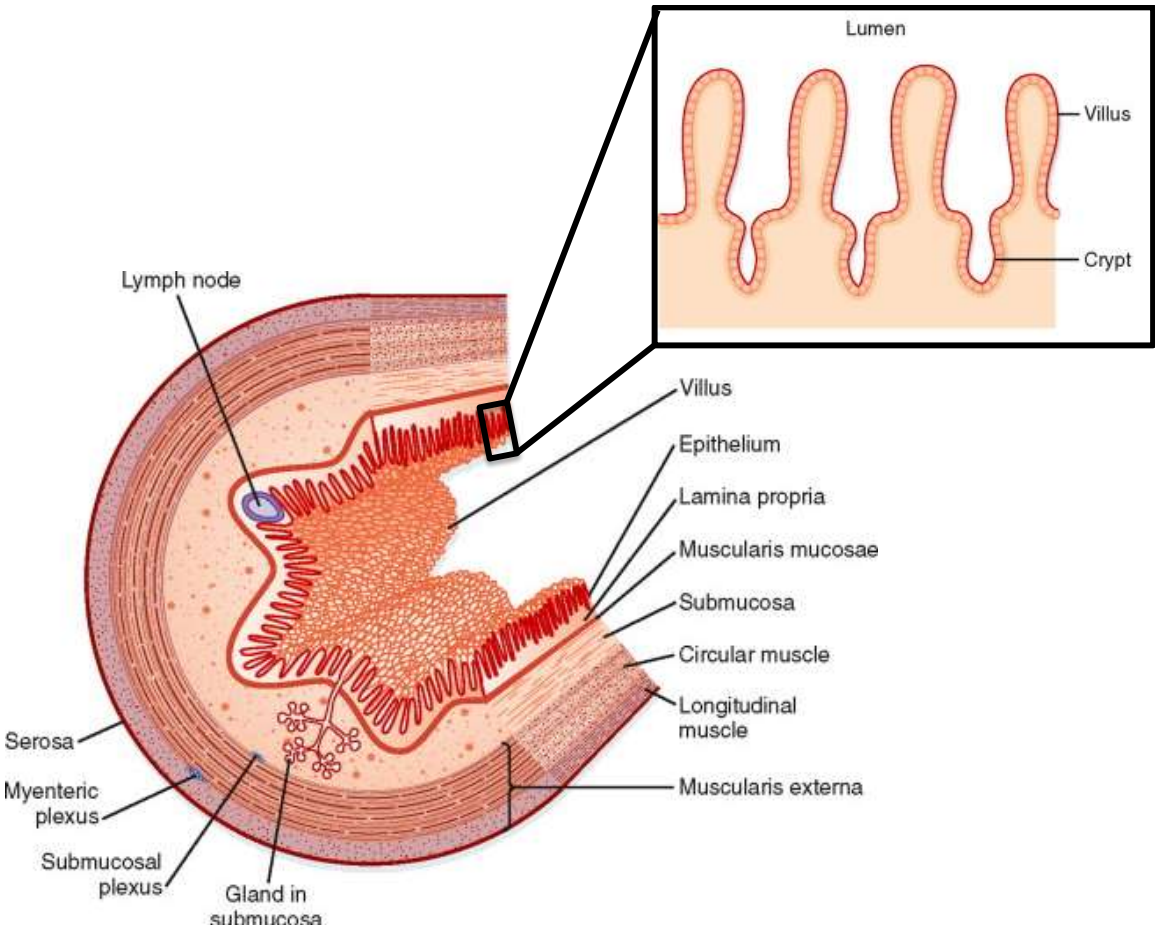


Fig. 1.1. Structure of the small intestine (Koeppen and Stanton, 2010).

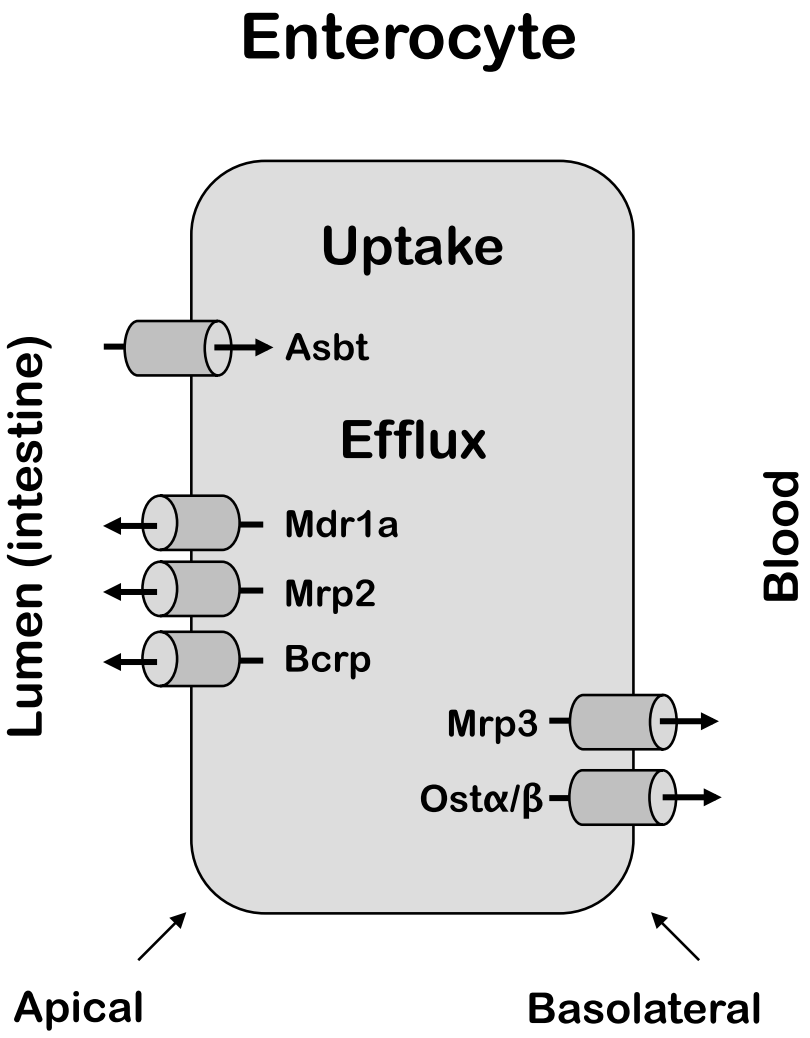


Fig. 1.2. Localization of transporters in the intestine.

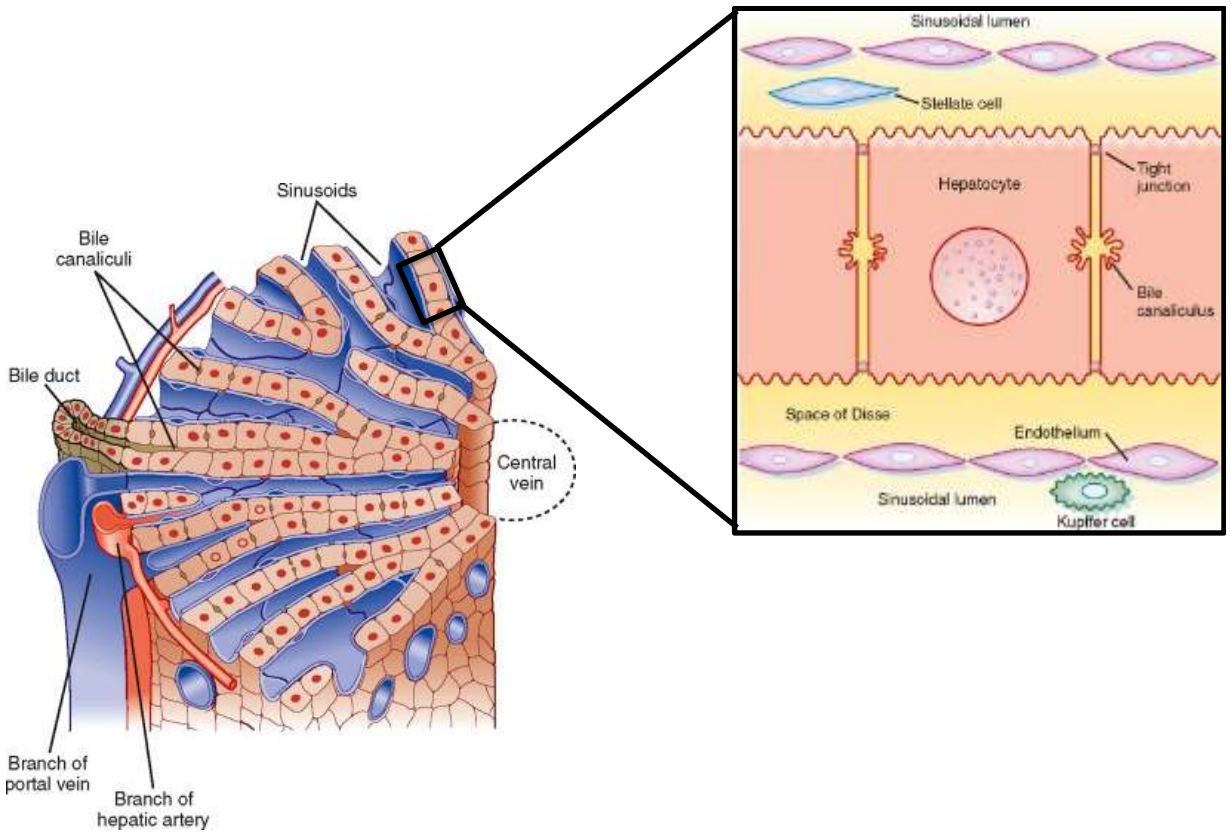


Fig. 1.3. Structure of the liver (Koeppen and Stanton, 2010).

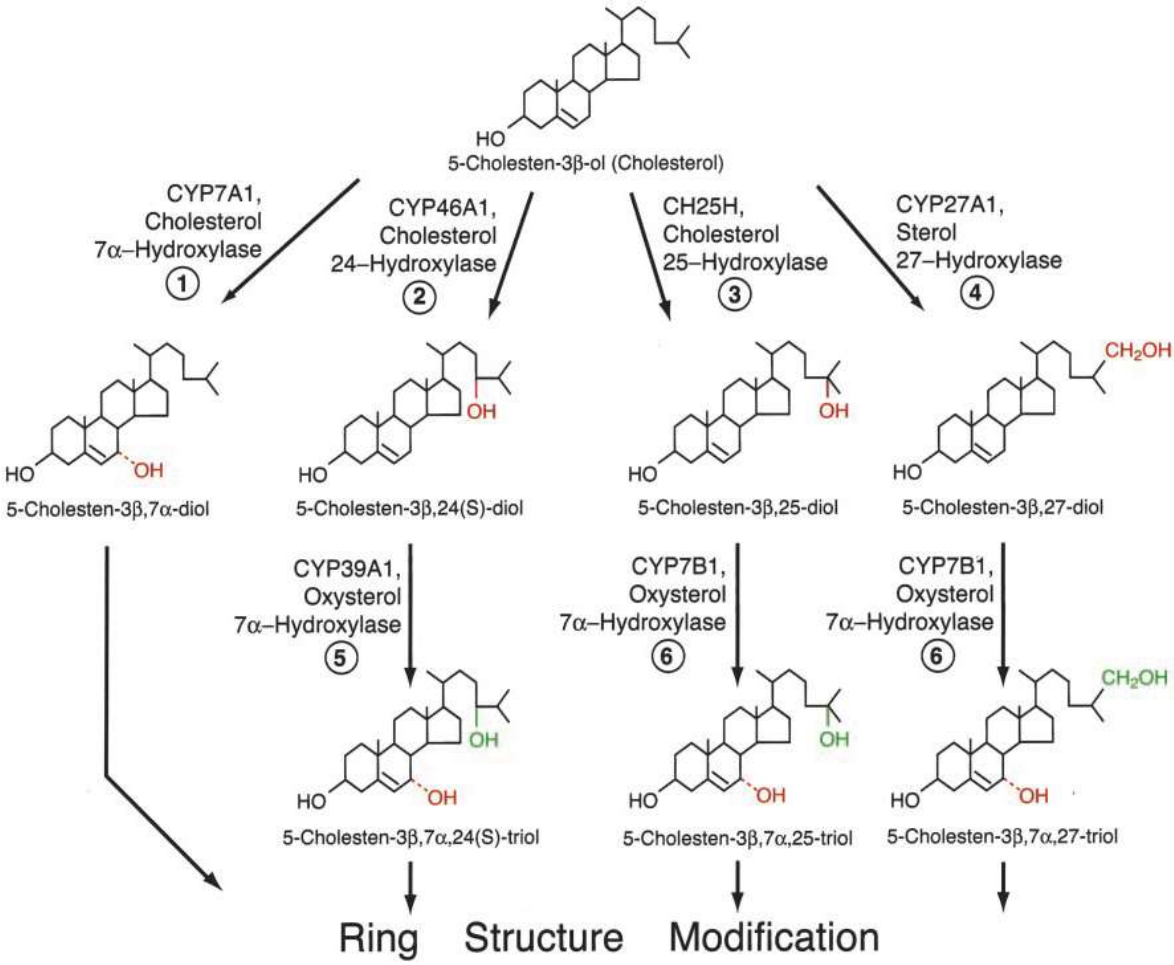


Fig. 1.4. Bile acid synthesis as regulated by cytochrome P450s (Russell, 2003).

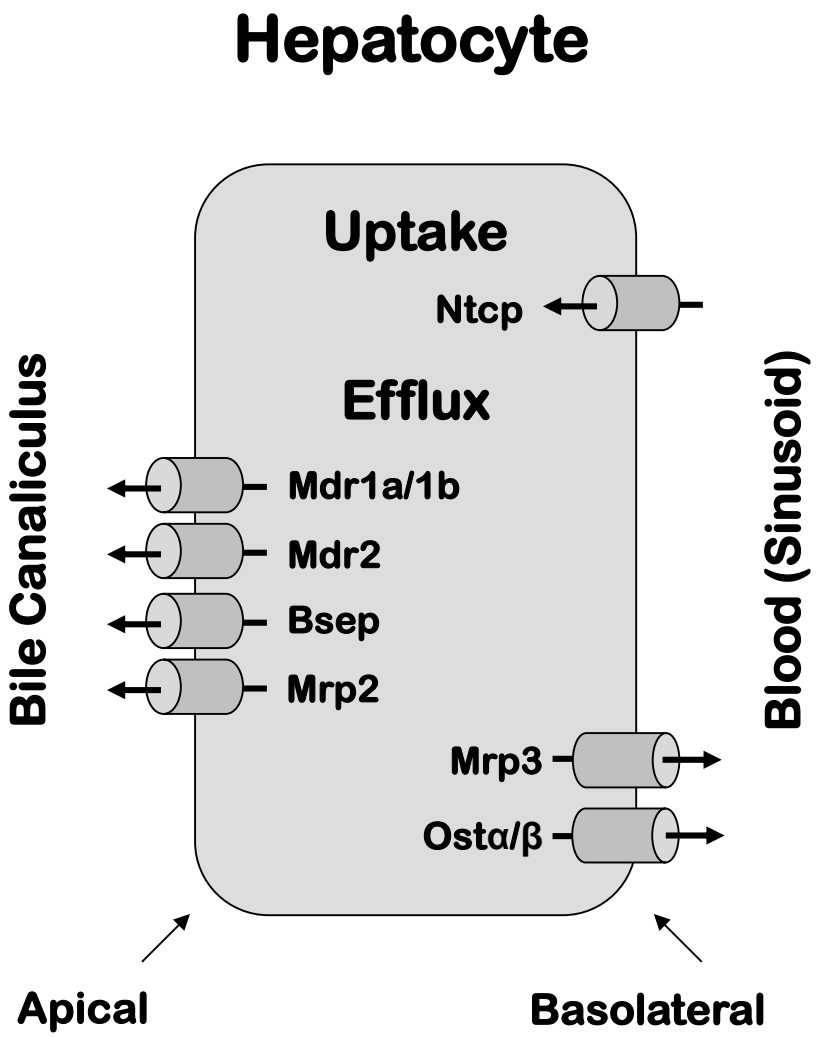


Fig. 1.5. Localization of transporters in the liver.

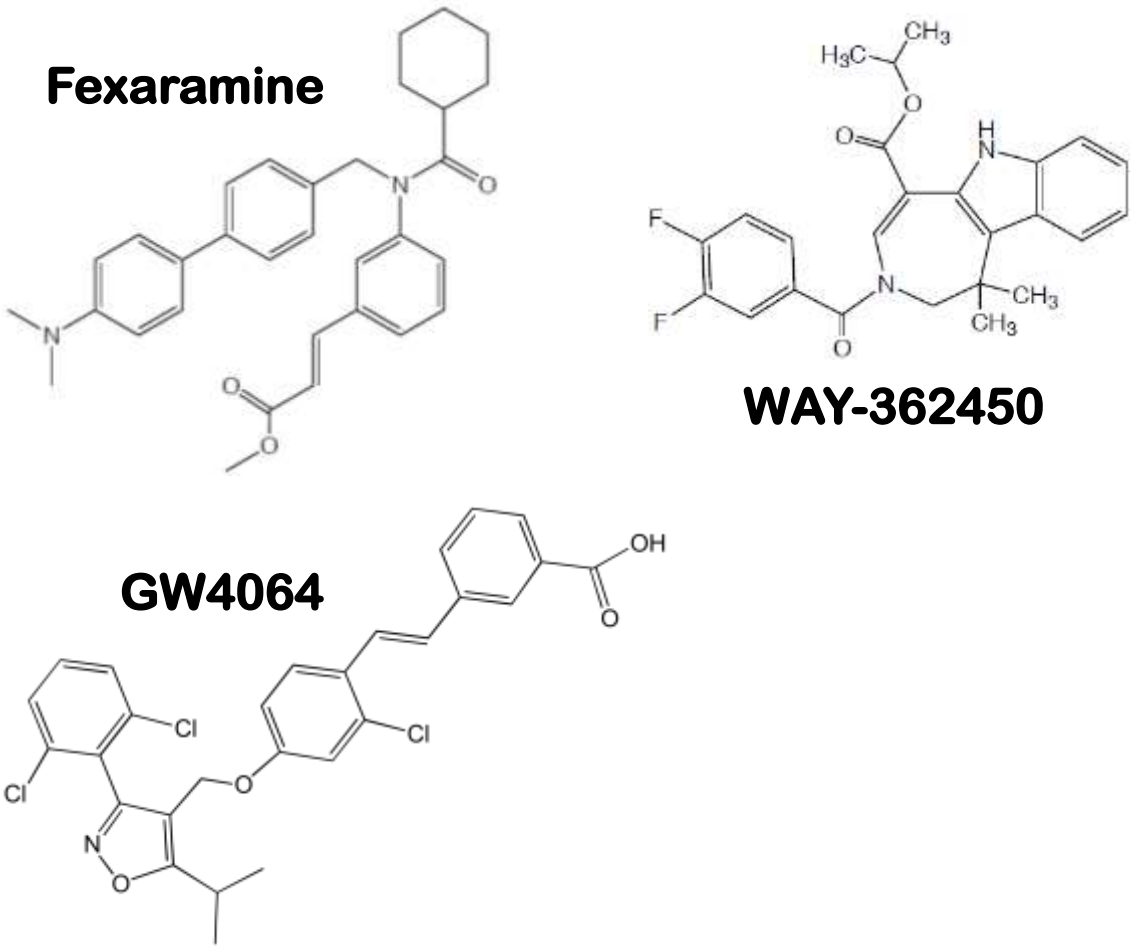


Fig. 1.6. Chemical structures of pharmacological agonists of FXR/Fxr.

Genetic defects associated to ICP				
Canalicular Transporter	Chromosomal Locus	Biochemical/Histologic Characteristics	Functional Defect	Clinical Spectrum
ATP8B1 (FIC1)	18q 21–22	High serum bile salts; low GGT/ bland cholestasis with coarse and granular bile	Abnormal excretion of aminophospholipids; downregulation of FXR	ICP, PFIC1, BRIC1, Byler disease
ABCB11 (BSEP)	2q24	High serum bile salts; low GGT/ portal tract fibrosis; bile duct proliferation	Abnormal bile acid secretion	ICP, Byler syndrome, PFIC2, BRIC2, drug-induced cholestasis, transient neonatal cholestasis
ABCB4 (MDR3)	7q21	High serum bile salts; elevated GGT/fibrosis, vanishing bile duct syndrome; low phospholipids in bile	Defect in phosphatidylcholine floppase	ICP, PFIC3, LPAC, neonatal cholestasis, drug-induced cholestasis
ABCC2 (MRP2)	10q24	High serum conjugated bilirubin/ black liver pigmentation	Alteration in canalicular transport of conjugated metabolites	ICP, Dubin–Johnson syndrome
NR1H4 (FXR)	12q23.1	High serum bile salts	Altered homeostasis of BSEP and MDR3	ICP, familial gallstone disease, idiopathic infantile cholestasis
FGF19	11q13.3	High serum bile salts	Abnormality in bile acid transport	ICP, Bile acid malabsorption

Abbreviations: BRIC, benign recurrent cholestasis; BSEP, bile salt export pump; FIC, familial intrahepatic cholestasis; FXR, farnesoid X receptor; GGT, gamma-glutamyl transferase; LPAC, low phospholipid cholestasis; MDR, multidrug resistance; MRP, multidrug resistance protein; PFIC, progressive familial intrahepatic cholestasis.

Table 1.1. Genetic variants associated with ICP (Floreani and Gervasi, 2016).

CHAPTER 2: DIFFERENTIAL REGULATION OF INTESTINAL EFFLUX TRANSPORTERS BY PREGNANCY AND STEROID HORMONES

Jamie E Moscovitz[a], Gabriel Yarmush[a], Guadalupe Herrera-Garcia[b], Grace L Guo[a,c], Lauren M Aleksunes[a,c]

[a] Department of Pharmacology and Toxicology, Rutgers University Ernest Mario School of Pharmacy, 170 Frelinghuysen Rd., Piscataway, NJ 08854, USA

[b] Department of Obstetrics and Gynecology, Rutgers-Robert Wood Johnson Medical School, 1 Robert Wood Johnson Place, New Brunswick, NJ 08901, USA

[c] Environmental and Occupational Health Sciences Institute, 170 Frelinghuysen Rd., Piscataway, NJ 08854, USA

2.1 Abstract

The small intestine represents the interface where nutrients and xenobiotics are absorbed from the gastrointestinal tract into the body. The nuclear receptors, Farnesoid X receptor (Fxr) and Pregnane X receptor (Pxr), regulate the transcription of key metabolizing enzymes and transporters in the intestines. The objective of this study was to profile the mRNA and protein expression of Fxr- and Pxr-regulated efflux transporters in pregnant mice. It was hypothesized that these signaling pathways would be repressed in response to high circulating concentrations of steroid hormones late in pregnancy. To test this, ilea were collected from time-matched virgin and pregnant mice on gestation days 14, 17 and 19, when sex hormone levels are highest. Ilea from pregnant mice on days 14 and 17 exhibited suppression of Fgf15 and Cyp3a11 mRNAs, which are prototypical target genes for Fxr and Pxr, respectively. An overall reduction in the expression of apical efflux transporters including Mdr1, Mrp2 and Bcrp was observed in pregnant mice. To assess the ability of individual steroid and placental hormones to alter intestinal nuclear receptor signaling, transporter mRNA expression was quantified in LS174T colon adenocarcinoma cells transfected with FXR. *In vitro* data demonstrate that progestins at concentrations relevant to the third trimester of human pregnancy reduce CYP3A4, MDR1 and MRP2 mRNA expression by 30 to 40%. In conclusion, Pxr/PXR-regulated efflux transporter expression was down-regulated in both pregnant mice and in intestinal cells treated with progestins. These data suggest a role for PXR-mediated progesterone effects on the molecular adaptations of the intestine during pregnancy.

Abbreviations

Cyp, cytochrome P450; Bcrp, breast cancer resistance protein; Fgf, fibroblast growth factor; Fxr, farnesoid X receptor; I-babp, ileal bile acid binding protein; Mdr, multidrug resistance protein; Mrp, multidrug resistance-associated protein; Ost, organic solute transporter; PM5S, epiallopregnanolone-sulfate; Pxr, pregnane x receptor; Shp, small heterodimer partner

2.2 Introduction

The small intestine represents the interface where nutrients are absorbed of into the body. Importantly, intestinal absorption can be a rate-limiting step for the uptake of orally-ingested chemicals and drugs. Several xenobiotic efflux transporters are located on the apical surface of enterocytes and can decrease xenobiotic bioavailability. These transporters include the multidrug resistance transporter, human MDR1/mouse Mdr1a (Ujhazy et al., 2001; Rost et al., 2002), the multidrug resistance-associated protein, MRP2/Mrp2 (Mottino et al., 2000; Rost et al., 2002), and the breast cancer resistance protein, BCRP/Bcrp (Maliepaard et al., 2001; Jonker et al., 2002). These transporters export a wide range of substrates and can reduce the amount of chemical that reaches the systemic circulation. Additional intestinal transporters, including MRP3/Mrp3 (Rost et al., 2002; Scheffer et al., 2002; Mutch et al., 2004) and the organic solute transporter heterodimer, OST/Ostα/β (Dawson et al., 2005; Rao et al., 2008), are tasked with secreting endogenous and exogenous compounds from the enterocyte into the portal circulation, where they then travel to the liver.

It has been reported that gastrointestinal motility is reduced in humans with advancing gestation, leading to an increase in gastric residence time and prolonged opportunity for nutrient uptake (Parry et al., 1970). This adaptation has also been observed in pregnant mice, pseudopregnant mice, and male rats treated with progesterone, suggesting that similar to humans, this hormone plays a key regulatory role (Datta et al., 1974; Wald et al., 1981; Wald et al., 1982; Liu et al., 2002). Prior studies have demonstrated the ability of steroid hormones to specifically influence hepatic drug metabolizing enzyme and transporter expression (reviewed in Jeong, 2010). In pregnancy, estradiol and progesterone reach peak concentrations of 0.1 and 1 μM (Jeong, 2010), respectively. Recently, levels of progesterone metabolites were detected in healthy pregnant women

at concentrations of 5-7 µM (non-pregnant value 0.08 µM) (Abu-Hayyeh et al., 2010; Abu-Hayyeh et al., 2013b). While the effect of these hormones on metabolism and transport in the liver has been studied extensively *in vivo* and *in vitro*, little data exists on their impact in the intestine.

The farnesoid X receptor (FXR/Fxr) and pregnane X receptor (PXR/Pxr) regulate bile acid homeostasis, in addition to genes that metabolize and excrete xenobiotics. Both nuclear receptors are highly expressed in the liver and small intestine, and respond directly or indirectly to hormone exposure. In the intestine, Fxr regulates fibroblast growth factor 15 (Fgf15), small heterodimer partner (Shp) and intestinal bile acid binding protein (I-babp), important factors regarding the trafficking of bile acids in enterocytes as well as decreasing hepatic bile acid synthesis (Oelkers and Dawson, 1995; Inagaki et al., 2005). Fxr also controls bile acid efflux into the portal circulation by direct transactivation of the Ostα/β genes (Frankenberg et al., 2006; Landrier et al., 2006). Pxr has many target genes involved in endobiotic and xenobiotic disposition, including the critical drug metabolizing enzyme human Cytochrome P450 (CYP)3A4/mouse Cyp3a11. Total CYP3A activity is increased in pregnant women as evidenced by enhanced metabolism of the drug midazolam in third trimester subjects (Hebert et al., 2008). However, quantification of the mouse ortholog Cyp3a11 revealed a down-regulation in livers of mice between gestation days 10 and 19, while the mRNA expression of other Cyp3a isoforms was induced (Aleksunes et al., 2013; Shuster et al., 2013). In addition to the enzyme Cyp3a11, Pxr can regulate the expression of apical and basolateral efflux transporters in the small intestine, including Mdr1, Mrp2/3 and Bcrp (Jigorel et al., 2006; Martin et al., 2008). Alterations to both the Fxr and Pxr signaling pathways may have critical implications for bile acid and xenobiotic disposition.

The LS174T cell line has been gaining use as an *in vitro* human intestinal model that more stably expresses most nuclear receptors, drug metabolizing enzymes, and xenobiotic transporters as compared to the Caco-2 cell line (Pfrunder et al., 2003). The regulation of MDR1 by PXR has been thoroughly explored in naïve LS174T cells, including modulation of MDR1 expression, localization and function by the PXR prototypical chemical inducer rifampicin and prototypical inhibitor ketoconazole (Kota et al., 2010). Moreover, the induction of FGF19, I-BABP and SHP gene expression by FXR agonists has been established in LS174T cells transiently transfected with the human *FXR* gene (LS174T-FXR) (Vaquero et al., 2013). While LS174T cells required transfection with the *FXR* gene to probe its activity, FXR expression in Caco-2 cells is dependent on a high degree of differentiation and extended time in culture (De Gottardi et al., 2004; Vaquero et al., 2013). Important to investigation of sex hormone-mediated regulation of disposition genes is the fact that naïve LS174T cells express the functional proteins of both the estrogen and progesterone receptors (Hendrickse et al., 1993).

To date, liver has been the primary tissue in studies aimed at understanding how pregnancy attenuates drug metabolism and transport. In addition, mechanistic studies have extensively described the interaction of steroid and placental hormones with hepatic enzymes and transporters. Recognizing that the intestine represents the interface for oral absorption, and is important to nutrient homeostasis and metabolism, there is a need to understand the molecular adaptations in intestinal nuclear receptor pathways during pregnancy. Further, it is critical to investigate potential mechanisms for altered transcriptional regulation by Fxr and Pxr. The purpose of the current study was to 1) determine the temporal expression of key ileal efflux transporters regulated by Fxr and Pxr during late pregnancy in mice and 2) identify potential sex hormones that mediate pregnancy-related changes in efflux transporters using an intestinal cell line.

2.3 Materials and methods

Chemicals

Unless otherwise specified, chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animal studies

Adult male and female C57BL/6 mice (strain 027) were purchased from Charles River Laboratories at 8-12 weeks of age (Wilmington, MA). All mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited animal care facility in temperature-, light- and humidity-controlled rooms. Studies were approved by the Rutgers University Institutional Animal Care and Use Committee, and were in accordance with national guidelines. A subset of female mice were mated overnight with male mice and checked for the presence of a vaginal sperm plug the next morning (designated gestation day 0). The remaining female mice were used as virgin controls. Mice had access to standard chow and water *ad libitum*. Small intestines were collected from virgin and pregnant time-matched mice on gestation days 14, 17 and 19. Small intestines were divided into three equal segments, representing the duodenum, jejunum and ileum, snap frozen in liquid nitrogen and stored at -80°C. Ileal fragments were utilized for all studies.

Cell culture and treatment

Human colon adenocarcinoma LS174T cells (ATCC, Manassas, VA) were cultured in 24-well plates with phenol red-free Minimum Essential Media (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum at 37ºC in an atmosphere containing 5% CO_2. On day 4, cells were transiently transfected with the pCMV-ICIS human *NR1H4/FXR* plasmid (LS174T-FXR, Open Biosystems, Huntsville, AL) (Li et al.,

2012) using Lipofectamine LTX according to the manufacturer's recommendations (ThermoFisher Scientific, Rockford, IL). On day 5, cells were cultured in media supplemented with 10% charcoal stripped fetal bovine serum and treated with vehicle (0.1% DMSO), 0.1 μM 17β-estradiol, 1 μM progesterone, 7 μM epiallopregnanolone-sulfate (PM5S, Steraloids, Newport, RI), 0.3 μM placental lactogen (NHPP, Torrance, CA), 13.3 nM testosterone, 7 pM growth hormone, 0.8 μM cortisol or 50 nM dehydroepiandrosterone (Steraloids), concentrations that represent third trimester levels in humans (Abu-Hayyeh et al., 2010; Jeong, 2010). Total RNA was collected from cells on day 6.

RNA isolation and quantitative PCR

Total RNAs were extracted from mouse ilea and LS174T-FXR cells using the RNeasy Mini Kit (Qiagen, Valencia, CA), and complementary DNA (cDNA) was generated using High Capacity cDNA Synthesis (Applied Biosystems, Foster City, CA). RNA purity and concentration were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Messenger RNA expression was quantified by real time-qPCR using the SYBR Green-based method (Applied Biosystems) for detection of amplified products. qPCR was performed in a 384-well plate format using the ViiA7 Real Time PCR machine (Life Technologies, Grand Island, NY). Ct values were converted to delta delta Ct values by adjusting to a reference gene (Ribosomal Protein L13A, RPL13A/Rpl13a) (Livak and Schmittgen, 2001). Specific forward and reverse primer sequences are listed in Table 2.1.

Western blot analysis

Ileal sections of the intestines were homogenized in sucrose-Tris buffer (pH=7.4-7.5) using the TissueLyser LT Adapter (Qiagen), per the manufacturer's protocol. Protein

concentrations were determined by BCA assay (Pierce Biotechnology, Rockford, IL). Forty micrograms of homogenate were loaded onto a SDS-PAGE gel (8%, Life Technologies). Semi-quantification of expression was determined using primary antibodies raised against Mdr1 (1:2000, C219, Abcam, Cambridge, MA), Mrp2 (1:600, Mrp_2III-5, Alexis), Bcrp (1:5000, BXP-53, Alexis), Ostα (1:1000) and Ostβ (1:1000) followed by incubation with a species-appropriate secondary antibody (Rao et al., 2008; Aleksunes et al., 2012). Primary antibodies for Ostα and Ostβ were supplied by Dr. Paul Dawson (Emory University, Atlanta, GA). The intensity of band luminescence was acquired using a FluorChem E System Imager (ProteinSimple, Santa Clara, CA). Na^+/K^+ ATPase (1:2000, sc-28800, Santa Cruz) was used as a loading control.

Statistical analysis

Data are presented as mean ± SE. Statistical analysis was performed using GraphPad Prism v6 (La Jolla, CA). mRNA and protein expression were analyzed by student's t-test (*in vivo* studies) or 1-way ANOVA followed by a Newman-Keul's multiple comparison post-hoc test (*in vitro* studies), to compare overall mean differences between groups. Significance was set at $p \leq 0.05$.

2.4 Results

Expression of intestinal signaling pathways in pregnant mice.

Similar to previous reports analyzing livers from pregnant mice (Milona et al., 2010a; Aleksunes et al., 2013), the mRNA expression of the nuclear receptor, Fxr, was unchanged in ilea on gestation days 14 through 19 (Fig. 2.1). Though Fxr expression was unchanged, all three of its target genes, including Fgf15, I-babp and Shp were suppressed on gestation days 14 and 17 by 50-95%, but approached virgin control levels by gestation day 19. Pxr mRNA was slightly reduced by 25% in the ilea of mice on gestation day 14. Likewise, the prototypical Pxr target gene, Cyp3a11, was down-regulated at all three gestational time points by 75 to 85%.

Expression of intestinal efflux transporters in pregnant mice.

Efflux transporter mRNA and protein expression profiles varied across time points in pregnant mice. Interestingly, there was a down-regulation of the luminal efflux transporters Mdr1a, Mrp2 and Bcrp (Fig. 2.2). There was a trend for decreased Mdr1a mRNA on gestation days 14 and 17. Likewise, Mdr1 protein expression was suppressed on gestation day 17 by 70% when compared to virgin controls. Mrp2 mRNA expression was significantly reduced by 60%, 70% and 45% on gestation days 14, 17 and 19, respectively. Both Bcrp mRNA and protein expression were decreased 30% and 55%, respectively, on gestation day 17 compared to time-matched virgin controls.

Ileal expression of the basolateral efflux transporters Mrp3, Ostα and Ostβ were differentially regulated during gestation (Fig. 2.3). Mrp3 mRNA levels were enhanced 2-fold on gestation day 14, returning to control levels on days 17 and 19. Ostα mRNA expression tended to be decreased on gestation day 17 by 40%, though mRNA of its heterodimerizing partner, Ostβ**,** steadily increased throughout gestation, with a noteable

1.6-fold up-regulation on gestation day 19. However, no changes in the expression of Ostα and Ostβ proteins were observed.

Expression of intestinal signaling pathways in sex hormone-treated LS174T-FXR cells.

Unlike in pregnant mice, FXRα mRNA was reduced by treatment with the individual hormones estradiol (20%), progesterone (45%) and PM5S (45%) in LS174T-FXR cells (Fig. 2.4). However, no reduction in downstream target gene expression (FGF19, I-BABP, SHP) was noted. CYP3A4, the prototypical target gene of human PXR and ortholog of Cyp3a11, was reduced by 30% with PM5S treatment. Likewise, MDR1 and MRP2 transporter mRNAs were down-regulated by 30-40% in the presence of either progesterone or its metabolite PM5S (Fig. 2.5). Alternatively, BCRP was up-regulated 40% by progesterone. In LS174T-FXR cells, placental lactogen induced MDR1 (2.7-fold) and BCRP (1.6-fold) and down-regulated MRP2 and MRP3 (35%) (Fig. 2.6). The androgens testosterone and dehydroepiandrosterone, as well as cortisol and pituitary growth hormone did not alter efflux transporter expression in LS174T-FXR cells (Fig. 2.6).

2.5 Discussion

This study provides the first comprehensive analysis of Fxr- and Pxr-mediated signaling in the intestines of pregnant mice and provides data regarding the potential for different sex hormones to mediate transcriptional changes. Suppression of the prototypical target genes Fgf15 and Cyp3a11 in the ilea of pregnant mice suggests that the signaling of Fxr and Pxr are reduced in late gestation, particularly on gestation days 14 and 17. An overall reduction in expression of Fxr target genes, including I-babp and Shp, as well as apical efflux transporters including Mdr1, Mrp2 and Bcrp was observed. Interestingly, a compensatory induction of Mrp3 occurred on gestation day 14. Protein expression of transporters Mdr1 and Bcrp largely mirrored pregnancy-related changes in mRNA levels.

It has been demonstrated that different regions of the intestines have unique expression profiles of nuclear receptors (Modica et al., 2010). The colon more highly expresses PXR/Pxr, while the ileum comparatively expresses higher levels of FXR/Fxr (Lax et al., 2012; Modica et al., 2012). Currently, there are no models available to study the human ileum *in vitro*, though primary epithelium isolated from the duodenum and jejunum are becoming commercially available. Unfortunately, the validity of primary enterocytes to study drug metabolizing enzymes and transporters has not been thoroughly tested. A previous report using the colon LS174T cell line demonstrates that genes regulated by FXR (FGF19, I-BABP and SHP) are not inducible by treatment with the FXR agonists chenodeoxycholic acid or GW4064 (Vaquero et al., 2013). However, transfection with the human FXR plasmid (LS174T-FXR cells) resulted in induction of FXR target genes with agonist treatment. In preliminary studies, transient transfection of LS174T cells with the *FXR* gene increased FXR mRNA levels from baseline (LS174T Ct values: 28-30 and LS174T-FXR Ct values: 11-13) (data not shown). Transient transfection did not alter baseline mRNA levels of any FXR or PXR target genes (data not shown). Therefore, in

the current study, LS174T-FXR cells were utilized to 1) recapitulate nuclear receptor enrichment typically observed in the ileum (Modica et al., 2010) and 2) permit the study of the effects of candidate hormones on both FXR and PXR pathways in the same *in vitro* system.

Several reports suggest that the size of the intestines and absorptive area of the small intestine are increased in pregnant rodents (Cripps and Williams, 1975; Prieto et al., 1994; Sabet Sarvestani et al., 2015). Therefore, it is possible that reduced expression of mRNA or protein may still yield the same total number of transporters in the intestinal tract. Nevertheless, down-regulation of metabolism and transport genes analyzed in mice was largely observed on gestation day 14-17, with resolution to virgin control levels by gestation day 19. The noted expression pattern across gestation time points mirrors the reported peak in progesterone levels from gestation day 14-17 and decline to parturition in mice (McCormack and Greenwald, 1974). Gene expression profiling of efflux transporters in LS174T-FXR cells treated with candidate hormones revealed that progestins down-regulated MDR1 and MRP2, and up-regulated BCRP. Similar regulation of MRP2 (reduction) and BCRP (induction) was observed with placental lactogen treatment. However, placental lactogen-treated cells additionally exhibited induction of MDR1 and repression of MRP3. Though data from *in vivo* pregnancy studies cannot be directly compared to that of *in vitro*, the transporter regulation by progestins and placental lactogen support the restoration of Mdr1a expression, but continued repression of Mrp2 expression observed in mice on gestation day 19.

While this is the first study to perform a profiling of endogenous and xenobiotic efflux transporters throughout late gestation in the ileum of mice, several studies have been conducted in mice that highlight changes in their expression in the intestine during

pregnancy. Previously published data suggest that mouse Fgf15 levels in the terminal ileum were unchanged in pregnancy (Milona et al., 2010a). However, Fgf15 mRNA levels were only quantified on gestation day 18, and the current study demonstrates that levels are returning to those observed in virgin controls by this time point (Fig. 2.1). Additional time course studies conducted in mice revealed that Cyp3a mRNAs, including Cyp3a11, in both the proximal and distal intestine were unchanged on gestation days 10, 15 and 19 (Zhang et al., 2008). The methods indicate that small intestines were only cut into two segments in this study (proximal and distal); suggesting that quantification of enzymes in the distal portion could be the combination of expression in both the jejunum and ileum, which may differ. In a prior study, Bcrp mRNA was induced in the small intestine of mice on gestation day 10, after which time expression returned to control levels (Wang et al., 2006). However, no change in protein expression was observed between pregnant and virgin mice (Wang et al., 2006). It should be noted that the current study utilized C57BL/6 mice, whereas aforementioned analyses were performed in FVB mice, which could contribute to the observed differences. These discrepancies between reports further support the importance of systematically evaluating time-dependent changes in xenobiotic and bile acid pathways in specific regions of the small intestine of pregnant dams.

Transactivation of xenobiotic disposition genes by progesterone and estradiol has been shown to be dose-dependent (Ekena et al., 1998; Attardi et al., 2007). Therefore, concentrations of steroid and placental hormones similar to those observed in the third trimester of pregnancy were used in cell culture treatments. The interaction of pregnenolone, as well as its metabolite, progesterone, with the ligand binding domain of PXR was identified as early as the 1990s (Kliewer et al., 1998). Progesterone activated PXR with an EC_{50} of 10 µM in CV-1 kidney cells transfected with the PXR expression

plasmid (Kliewer et al., 1998). Progesterone metabolites have been shown to alter Pxr function in pregnancy. Interestingly, pregnant mice lacking Pxr did not exhibit the adaptations in vascular function typically observed in wild-type damns (Hagedorn et al., 2007). Further, authors showed that treatment of virgin Pxr-null mice with a subcutaneous implant of the progesterone metabolite 5β-dihydroprogesterone for 7 days did not mimic the pregnancy-related adaptations in vascular tone that were observed in virgin wild-type mice, pointing to Pxr-mediated signaling of progesterone metabolites (Hagedorn et al., 2007). In the current study, repression of PXR/Pxr target genes (Cyp3a11/CYP3A4, Mdr1a/MDR1, Mrp2/MRP2) was detected in both pregnant mice and intestinal cells treated with progesterone and its metabolite. It is hypothesized that progesterone is interfering with PXR/Pxr signaling, resulting in reduced expression of important downstream xenobiotic enzymes and transporters. Linear regressions for transporter expression fold change and progesterone concentrations were generated for gestational day 14-19 utilizing previously reported serum hormone levels (McCormack and Greenwald, 1974). While correlations were not significant, as determined by a Spearman rank analysis, strong correlation coefficients of 0.97, 0.76 and 0.82 were calculated for Mdr1a, Mrp2 and Bcrp, respectively (data not shown). By comparison, correlation coefficients for 17β-estradiol and Mdr1a, Mrp2 and Bcrp were 0.72, 0.12 and 0.16, respectively. Further studies are needed to determine whether there is a unique concentration-dependent relationship between progesterone and PXR; such that physiologically relevant concentrations of progesterone (<5 μM) and its metabolite PM5S can inhibit PXR, while high concentrations can activate signaling as previously reported (Blumberg et al., 1998; Kliewer et al., 1998).

The objective of this study was to assess the expression profiles of intestinal bile acid and xenobiotic disposition genes regulated by Fxr and Pxr during late pregnancy, and

identify candidate sex hormones that may be responsible for altered nuclear receptor signaling. Candidate hormones were selected based on evidence in the literature of signaling through nuclear receptor pathways, and influence on drug metabolizing enzyme and transporter expression. Our findings suggest that there is interference with both Fxr and Pxr signaling during pregnancy, as evidenced by suppression of genes and proteins in both pathways. *In vitro* data support these *in vivo* observations indicating that interference with PXR/Pxr transcriptional regulation, likely by high progestin concentrations, may play a greater role in molecular adaptations of the intestine during pregnancy. Future studies should 1) assess the impact of mixtures of steroid and placental hormones on hepatic and intestinal drug metabolizing enzymes and transporters, as it is possible they can modify expression through shared nuclear receptor signaling pathways and 2) investigate functional modifications to the luminal efflux transporters that contribute to the extent of intestinal drug absorption for consideration during pharmacokinetic profiling of drugs in pregnant women.

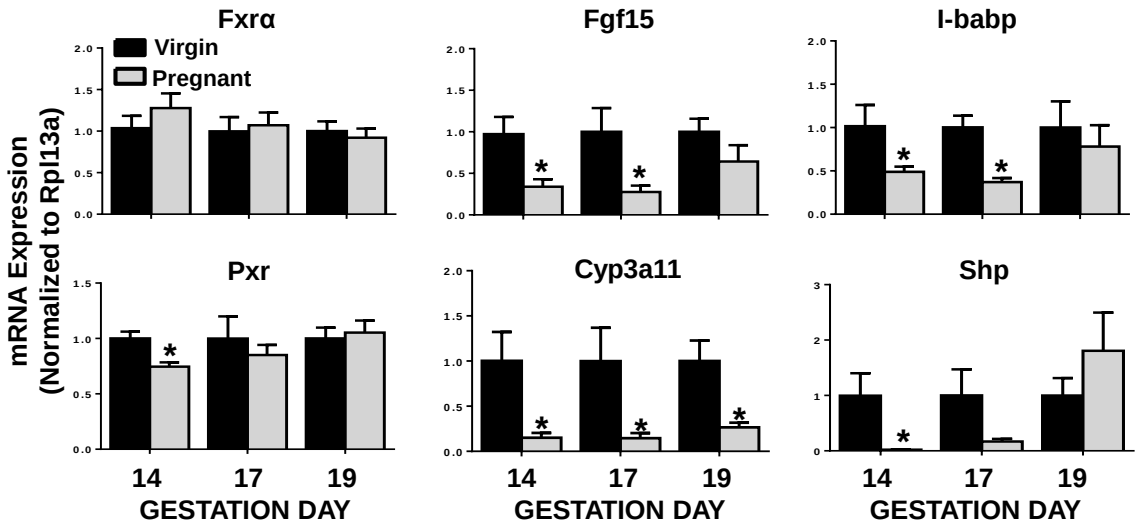


Fig. 2.1. Gene expression of nuclear receptor signaling pathways in pregnant mice. mRNA expression was quantified in virgin and pregnant mice on gestation days 14, 17 and 19. Data were normalized to virgin controls (set to 1.0) at each time point and presented as mean relative expression ± SE (n=5-6). Black bars represent virgin mice, and grey bars represent pregnant mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with time-matched virgin controls.

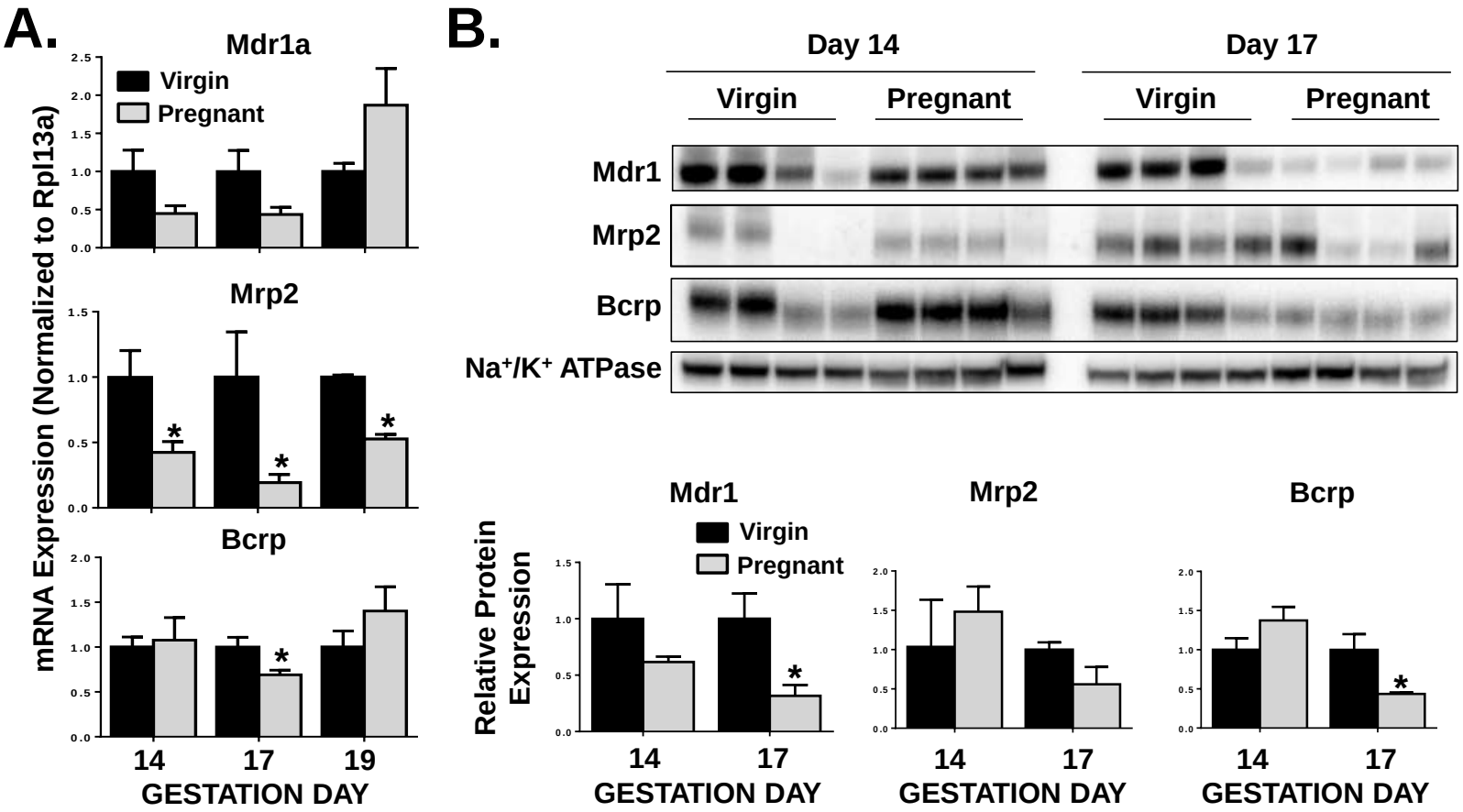

A.
Mdr1a
Virgin
Pregnant
Mrp2
Bcrp
mRNA Expression (Normalized to Rpl13a)
14
17
19
GESTATION DAY
B.
Day 14
Day 17
Virgin
Pregnant
Virgin
Pregnant
Mdr1
Mrp2
Bcrp
Na+/K+ ATPase
Mdr1
Mrp2
Bcrp
Virgin
Pregnant
Relative Protein Expression
14
17
GESTATION DAY

Fig. 2.2. Expression of intestinal apical efflux transporters in pregnant mice. (A) mRNA and (B) protein expression were quantified in virgin and pregnant mice on gestation days 14, 17 and 19 (mRNA) or gestation days 14 and 17 (protein). Western blot staining was performed using whole ileal homogenates. Representative images are shown. Band density was semi-quantified. Data were normalized to virgin controls (set to 1.0) at each time point and presented as mean relative expression ± SE (n=5-6 for mRNA, n=4 for protein). Black bars represent virgin mice, and grey bars represent pregnant mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with time-matched virgin controls.

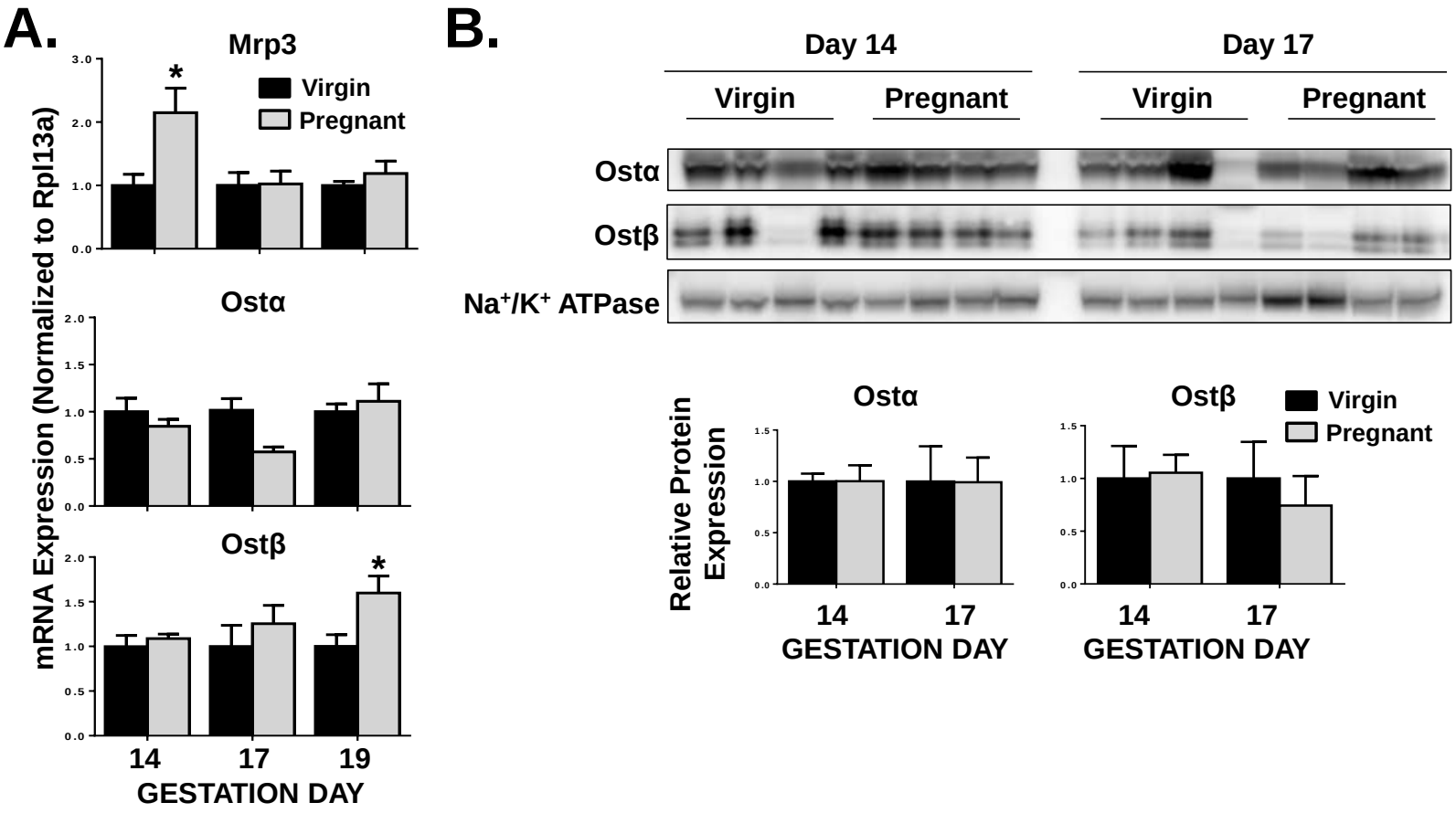

A.
Mrp3
Virgin
Pregnant
*
Ostα
Ostβ
*
mRNA Expression (Normalized to Rpl13a)
3.0
2.0
1.0
0.0
2.0
1.5
1.0
0.5
0.0
14
17
19
GESTATION DAY
B.
Day 14
Day 17
Virgin
Pregnant
Virgin
Pregnant
Ostα
Ostβ
Na+/K+ ATPase
Relative Protein Expression
Ostα
Ostβ
Virgin
Pregnant
1.5
1.0
0.5
0.0
14
17
GESTATION DAY
14
17
GESTATION DAY

Fig. 2.3. Expression of intestinal basolateral efflux transporters in pregnant mice. (A) mRNA and (B) protein expression were quantified in virgin and pregnant mice on gestation days 14, 17 and 19 (mRNA) or gestation days 14 and 17 (protein). Western blot staining was performed using whole ileal homogenates. Representative images are shown. Band density was semi-quantified. Data were normalized to virgin controls (set to 1.0) at each time point and presented as mean relative expression ± SE (n=5-6 for mRNA, n=4 for protein). Black bars represent virgin mice, and grey bars represent pregnant mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with time-matched virgin controls.

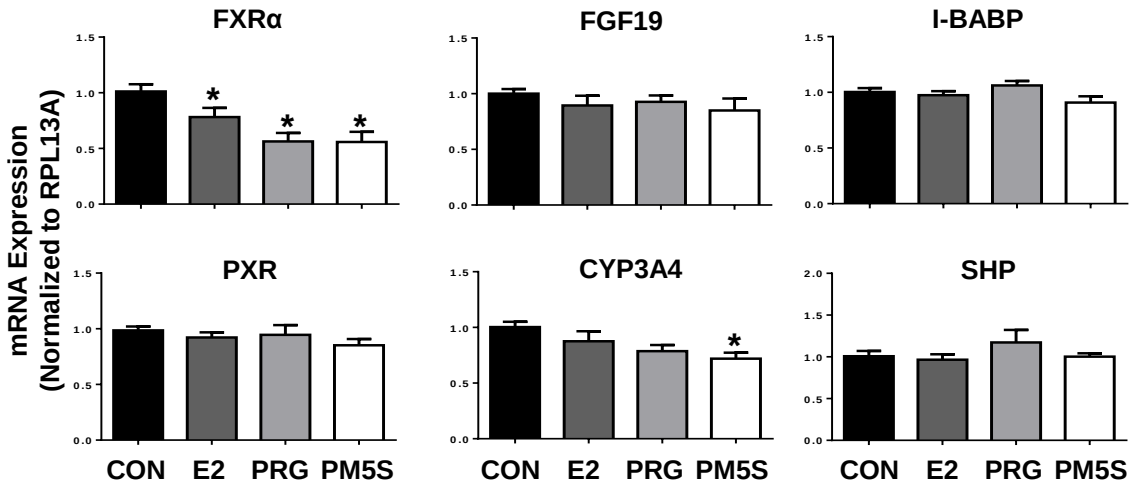


Fig. 2.4. Gene expression of nuclear receptor signaling pathways in intestinal cells treated with steroid hormones and metabolites. mRNA expression was quantified in LS174T-FXR cells treated with vehicle (CON, set to 1.0), estradiol (E2, 0.1 μM), progesterone (PRG, 1 μM) or epiallopregnanolone-sulfate (PM5S, 7 μM) for 24 hours. Data were normalized to vehicle and presented as mean relative expression ± SE (n=3 independent experiments). Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated cells.

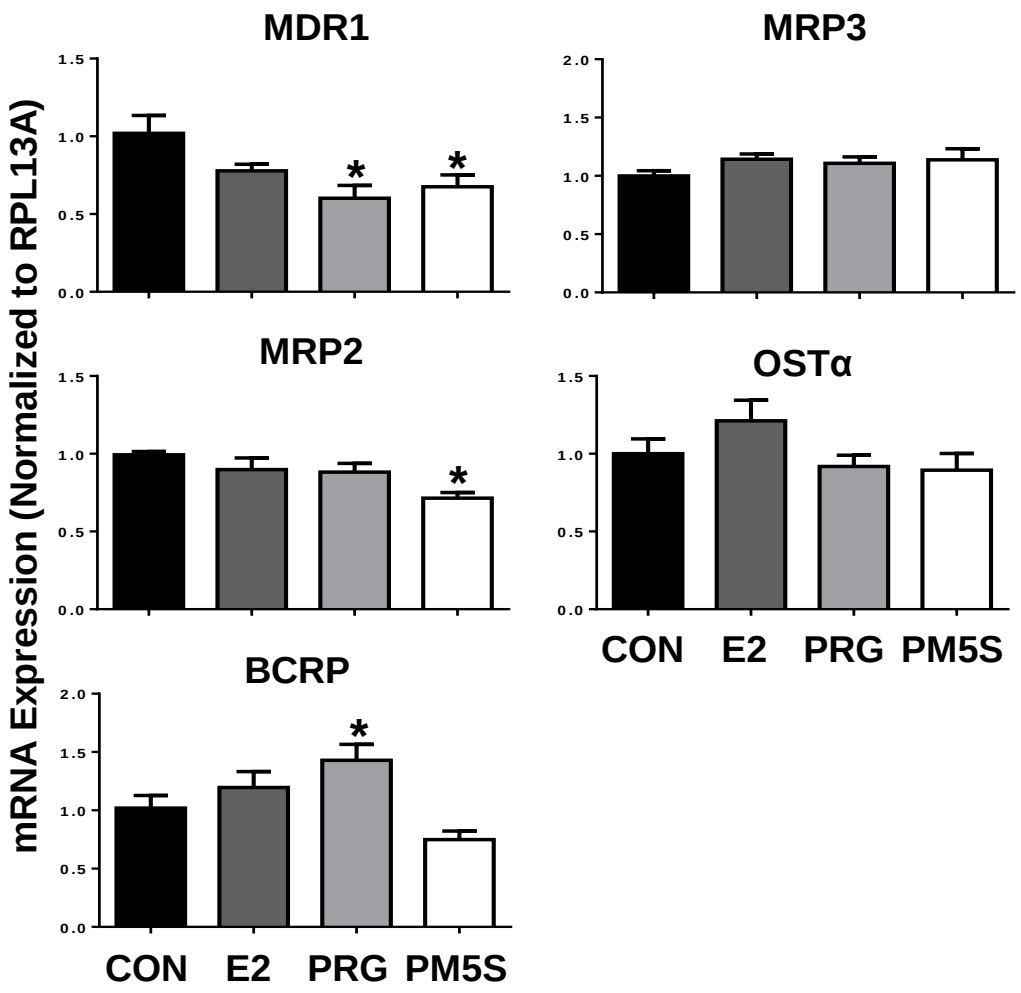


Fig. 2.5. Gene expression of efflux transporters in intestinal cells treated with steroid hormones and metabolites. mRNA expression was quantified in LS174T-FXR cells and treated with vehicle (CON, set to 1.0), estradiol (E2, 0.1 μM), progesterone (PRG, 1 μM) or epiallopregnanolone-sulfate (PM5S, 7 μM) for 24 hours. Data were normalized to vehicle and presented as mean relative expression ± SE (n=3 independent experiments). Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated cells.

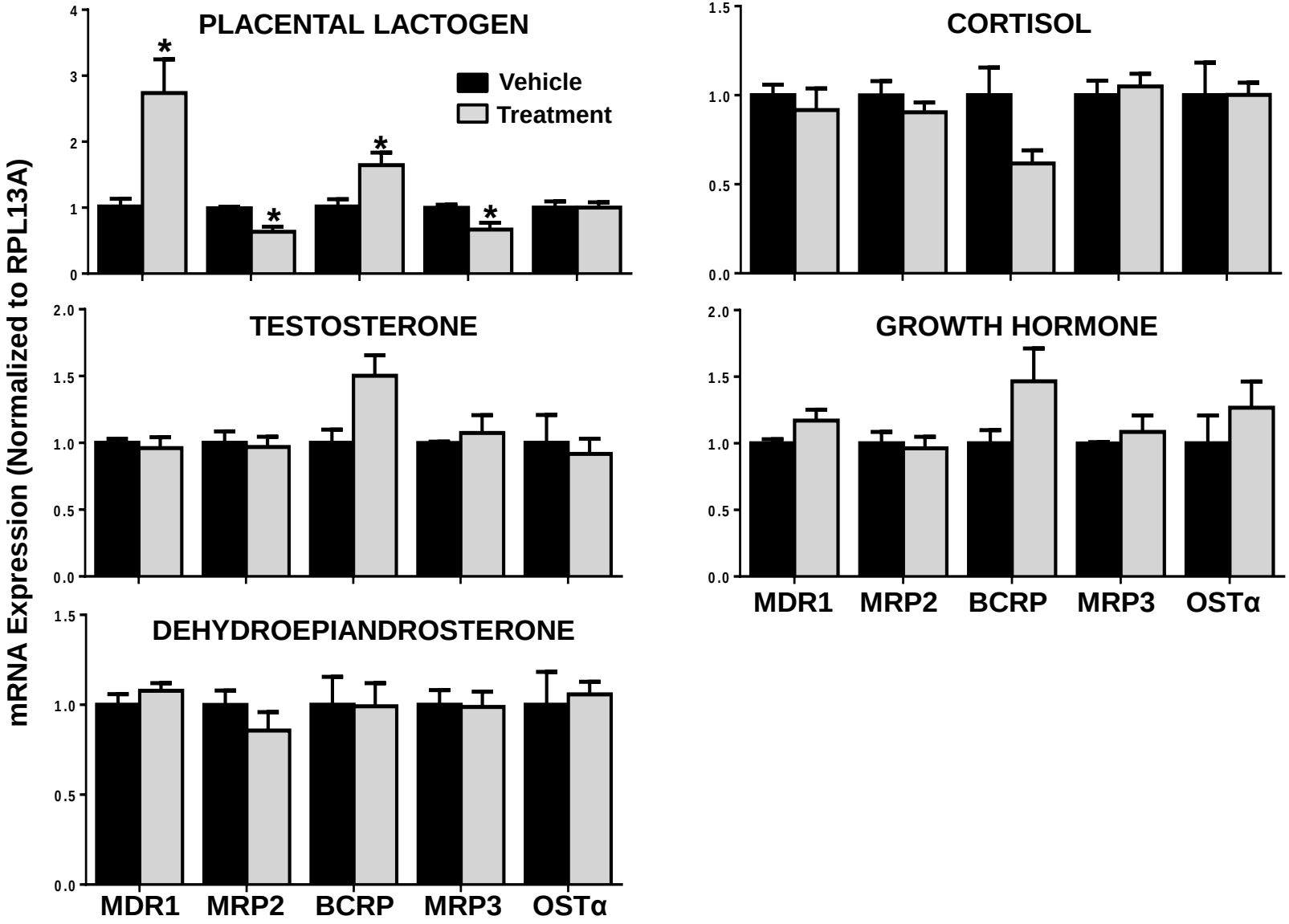

PLACENTAL LACTOGEN
Vehicle
Treatment
CORTISOL
TESTOSTERONE
GROWTH HORMONE
DEHYDROEPIANDROSTERONE
mRNA Expression (Normalized to RPL13A)
MDR1
MRP2
BCRP
MRP3
OSTα

Fig. 2.6. Gene expression of efflux transporters in intestinal cells treated with steroid and placental hormones. mRNA expression was quantified in LS174T-FXR cells treated with vehicle (set to 1.0), placental lactogen (6 μg/mL), testosterone (13.3 nM), dehydroepiandrosterone (50 nM), cortisol (0.8 μM) or growth hormone (7 pM) for 24 hours. Data were normalized to vehicle and presented as mean relative expression ± SE (n=3 independent experiments). Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated cells.

Table 2.1. qPCR primer sequences

Primer	Forward (5’ to 3’)	Reverse (5’ to 3’)
MOUSE		
Cyp3a11	CCCAGTGGGGATAATGAGTAA	CTTGCCTTTCTTTGCCTTCT
Fgf15	GCCATCAAGGACGTCAGCA	CTTCCTCCGAGTAGCGAATCAG
Fxrα	TCCGGACATTCAACCATCAC	TCACTGCACATCCCAGATCTC
I-babp	CCCCAACTATCACCAGACTTC	ACATCCCCGATGGTGGAGAT
Mdr1a	TGCCCCACCAATTTGACACCCT	ATCCAGTGCGGCCTGAACCA
Mrp2	AGCAGGTGTTCGTTGTGTGT	AGCCAAGTGCATAGGTAGAGAAT
Mrp3	CTGGGTCCCCTGCATCTAC	GCCGTCTTGAGCCTGGATAAC
Ostα	CACTGGCTCAGTTGCCATTT	GCATACGGCATAAAACGAGGT
Ostβ	GCAAACAGAAATCGAAAGAAGC	TCTGGCAGAAAGACAAGTGAT
Pxr	GCAGCATCTGGAACTACCAA	CTGGTCCTCAATAGGCAGGT
Rpl13a	CAAGAAAAAGCGGATGGTGG	TCCGTAACCTCAAGATCTGC
Shp	CGATCCTCTTCAACCCAGATG	AGGGCTCCAAGACTTCACACA
HUMAN		
CYP3A4	CACAAACCGGAGGCCTTTTGGTC	GTCTCTGCTTCCCGCCTCAGAT
FGF19	TCGGAGGAAGACTGTGCTTTC	CCTCTCGGATCGGTACACATTG
FXRα	CGCCTGACTGAATTACGGACA	TCACTGCACGTCCCAGATTTC
I-BABP	CTCCAGCGATGTAATCGAAA	CCCCCATTGTCTGTATGTTG
MDR1	TTGAAATGAAAATGTTGTCTGG	CAAAGAAACAACGGTTCGG
MRP2	AGCCATGCAGTTTTCTGAGGCCT	TGGTGCCCTTGATGGTGTGC
MRP3	CTTCCTGGTGACCCTGATCACCCT	TGCTGGATCCGTTTCAGAGACACA
OSTα	GGAGCACAGCTCTATGGATCA	TCAGGATGAGGAGAACCTGGA
PXR	ATCTCCTACTTCAGGGACTT	AGCTTCTTCAGCATGTAGTG
RPL13A	GGTGCAGGTCCTGGTGCTTGA	GGCCTCGGGAAGGGTTGGTG
SHP	ATCCTCTTCAACCCCGATGTG	GTCGGAATGGACTTGAGGGT

CHAPTER 3: RESTORATION OF ENTEROHEPATIC BILE ACID PATHWAYS IN PREGNANT MICE FOLLOWING SHORT TERM ACTIVATION OF FXR BY GW4064

Jamie E Moscovitz[a], Bo Kong[a], Kyle Buckley[a], Brian Buckley[b], Grace L Guo[a,b], Lauren M Aleksunes[a,b]

[a] Department of Pharmacology and Toxicology, Rutgers University Ernest Mario School of Pharmacy, 170 Frelinghuysen Rd., Piscataway, NJ 08854, USA

[b] Environmental and Occupational Health Sciences Institute, 170 Frelinghuysen Rd., Piscataway, NJ 08854, USA

3.1 Abstract

The farnesoid X receptor (Fxr) controls bile acid homeostasis by coordinately regulating the expression of synthesizing enzymes (Cyp7a1, Cyp8b1), conjugating enzymes (Bal, Baat) and transporters in the intestine (Asbt, Ostα/β) and liver (Ntcp, Bsep, Mrp3, Ostβ). Transcriptional regulation by Fxr can be direct, or through the ileal Fgf15/FGF19 and hepatic Shp pathways. Circulating bile acids are increased during pregnancy due to hormone-mediated disruption of Fxr signaling. While this adaptation enhances lipid absorption, elevated bile acids may predispose women to develop maternal cholestasis. The objective of this study was to determine whether short term treatment of pregnant mice with GW4064 (a potent FXR agonist), restores Fxr signaling to the level observed in virgin mice. Plasma, liver and intestines were collected from virgin and pregnant wild-type mice administered vehicle or GW4064 by oral gavage. Treatment of pregnant mice with GW4064 induced ileal Fgf15, Shp and Ostα/β mRNAs, and restored hepatic Shp, Bal, Ntcp, and Bsep expression back to vehicle-treated virgin levels. Pregnant mice exhibited 2.5-fold increase in Cyp7a1 mRNA compared to virgin controls, which was reduced by GW4064. Similarly treatment of female mouse primary hepatocytes with plasma isolated from pregnant mice induced Cyp7a1 mRNA by nearly 3-fold as compared to virgin plasma, which could be attenuated by co-treatment with either GW4064 or exogenous recombinant FGF19 protein. Collectively, these data demonstrate that deficient intestinal and hepatic Fxr function in pregnancy may be restored by pharmacological activation. This study provides the basis for a novel approach to restore bile acid homeostasis in patients with maternal cholestasis.

Abbreviations

Asbt, apical sodium-dependent bile acid transporter; Baat, bile acid CoA:amino acid N-acetyltransferase; Bal, bile acid CoA ligase; Bsep, bile salt export pump; Cyp, cytochrome P450; Fgf, fibroblast growth factor; Fxr, farnesoid X receptor; I-babp, ileal bile acid binding protein; ICP, intrahepatic cholestasis of pregnancy; Mdr, multidrug resistance protein; Mrp, multidrug resistance-associated protein; Ntcp, Na^+-taurocholate cotransporting polypeptide; Ost, organic solute transporter; Shp, small heterodimer partner

3.2 Introduction

Bile acids are required for the absorption of lipids and lipid-soluble vitamins from the intestine. During periods of high nutritional demand such as pregnancy, the body adapts to increase the size of the bile acid pool. While this is necessary for enhanced absorption of nutrients to support placental and fetal growth, hypercholanemia can lead to maternal cholestasis or a condition called intrahepatic cholestasis of pregnancy (ICP). ICP is observed in 0.5-2% of pregnancies in the United States, and carries with it an increased risk of fetal distress, spontaneous preterm delivery and stillbirth (Glantz et al., 2004; Geenes et al., 2014a; Henderson et al., 2014; Williamson and Geenes, 2014). Likewise, women with ICP are more likely to have concomitant gestational diabetes or pre-eclampsia, and may be predisposed to develop subsequent liver disease, such as hepatitis C, fibrosis and gallstones (Marschall et al., 2013; Wikstrom Shemer et al., 2013).

The synthesis, metabolism, and excretion of bile acids is tightly regulated by transcription factors, enzymes, transporters, and signaling mediators in the liver and intestine. Only 5% of bile acids are excreted into the feces each day. The remaining 95% are conserved through enterohepatic recirculation (Danielsson and Sjovall, 1975). Bile acids are synthesized in the liver via the classic (key enzymes cholesterol-7α-hydroxylase, Cyp7a1, and sterol 12α-hydroxylase, Cyp8b1) or alternative (key enzyme steroid 27-hydroxylase, Cyp27a1) pathways and released into the intestinal lumen to promote the absorbance of lipids and lipid-soluble vitamins. Bile acids are reabsorbed into enterocytes by the apical sodium-dependent bile acid transporter (Asbt). Bile acids are thought to be bound by intestinal binding proteins such as ileal bile acid binding protein (I-babp), and secreted into the portal circulation by the basolateral organic solute transporter alpha/beta (Ostα/β) heterodimer (Dawson et al., 2005). Portal bile acids are

then taken up into hepatocytes predominantly by the sinusoidal transporter Na^+-taurocholate cotransporting polypeptide (Ntcp). Removal of bile acids from the liver is mainly accomplished by the canalicular efflux transporters bile salt export pump (Bsep) and multidrug resistance-associated protein (Mrp) 2 (Keppler et al., 1997; Stieger et al., 2007). However, the sinusoidal efflux transporters Ostα/β and Mrp3 can also pump bile acids into the circulation. Sinusoidal efflux of bile acids into the blood can be enhanced and is critical for removing bile acids from the liver during cholestasis (Belinsky et al., 2005; Landrier et al., 2006; Teng and Piquette-Miller, 2007).

The farnesoid X receptor (Fxr) is a bile acid-activated transcription factor that belongs to the nuclear receptor superfamily. Fxr cooperatively regulates the expression of bile acid transporters including intestinal and hepatic Ostα/β, hepatic Ntcp and Bsep (Lu et al., 2000; Denson et al., 2001), as well as the inhibitory hepatic transcription factor small heterodimer partner (Shp) and the intestinal endocrine factor fibroblast growth factor (Fgf) 15 (FGF19 in humans). Fxr-mediated induction of Fgf15 in the intestine has emerged as a major pathway to suppress bile acid synthesis in the liver (Kong et al., 2012). In detail, Fxr activation induces Fgf15/19 in enterocytes, which is secreted into the portal vein and then binds to its cell surface receptor Fgfr4 on hepatocytes where it represses the expression of bile acid synthesizing genes (Cyp7a1 and Cyp8b1) (Inagaki et al., 2005; Yu et al., 2005). In response to bile acid binding in the liver, Fxr up-regulates the transcription of Shp, which works with Fgf15 to suppress the expression of Cyp7a1. Furthermore, Fxr transactivates the *Abcb11*/Bsep and *Slc51*/Ostα/β genes (Zhu et al., 2011; Kong et al., 2012). During pregnancy, elevated bile acid levels have been attributed to suppressed hepatic Fxr function by increased circulating levels of the steroid hormones estradiol and progesterone, and their metabolites, which results in enhanced synthesis and reduced hepatic uptake and secretion of bile acids (Abu-

Hayyeh et al., 2010; Milona et al., 2010a; Aleksunes et al., 2012; Abu-Hayyeh et al., 2013a).

Despite recent advancements in the ICP and bile acid fields, there are a number of knowledge gaps that remain. It is not known whether intestinal Fxr function is modified during pregnancy. Moreover, the potential to intervene and/or reverse the adaptive modifications to bile acid homeostasis has not been tested in pregnant animals. Therefore, the purposes of this study were to 1) characterize ileal Fxr functions during pregnancy in mice and 2) test the ability to overcome repressed Fxr functions using a selective Fxr agonist. Therefore, pharmacologic approaches to modulate Fxr activity *in vivo* and *ex vivo* were used to delineate the molecular mechanisms by which ileal and hepatic Fxr regulate bile acid homeostasis during pregnancy.

3.3 Materials and methods

Chemicals

Unless otherwise specified, chemicals were obtained from Sigma-Aldrich (St. Louis, MO). GW4064 was synthesized at the Chemical Discovery Laboratory at the University of Kansas (Lawrence, KS). Recombinant FGF19 protein was synthesized by the laboratory of Dr. Guo at Rutgers University according to a previous report (Kong and Guo, 2014).

Animal treatment

Adult male and female wild-type C57BL/6 mice 8-12 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). Female mice were mated overnight with male mice and checked for the presence of a vaginal sperm plug the next morning (designated gestation day 0). Additional female mice were used as virgin controls. Mice had access to standard chow and water *ad libitum*. On gestation days 13 and 14, pregnant females (n=5-6) and time-matched virgin females received two doses of vehicle (PBS with 1% Tween-80 and 1% methylcellulose, 10 mL/kg) or the Fxr synthetic agonist GW4064 (100 mg/kg, 10 ml/kg) 12 hours apart by oral gavage. Plasma, liver and intestines were collected from vehicle- and GW4064-treated virgin and pregnant mice 3 hours after the second dose on gestation day 14. Small intestines were divided into three equal segments, representing the duodenum, jejunum and ileum. Ileal fragments were utilized for all studies.

Liver and total body weights were recorded for female mice at the time of sacrifice. Tissues were collected within a 60 minute period in the morning (Zhang et al., 2011b), snap frozen in liquid nitrogen, and stored at -80°C. All mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited

animal care facility in temperature-, light- and humidity-controlled rooms. All animal studies were approved by the Rutgers University Institutional Animal Care and Use Committee, and were in accordance with national guidelines.

Primary hepatocyte culture

Freshly isolated female C57BL/6 mouse hepatocytes (n=3 donors in triplicate) were obtained from Triangle Research Labs (Research Triangle Park, NC). Hepatocytes were cultured in 12-well or 6-well plates with a Matrigel overlay, and shipped overnight in cold preservation media. Upon receipt, the preservation media was replaced with serum-free and phenol red-free Williams' E media. Cells were allowed to recover from shipping for 24 hours at 37°C in an atmosphere containing 5% CO_2. After recovery, the hepatocytes were treated with phenol red-free William's E media containing 20% pooled virgin or pregnant (gestation day 17 or greater) mouse plasma, in the presence of GW4064 (5 μM), recombinant FGF19 protein (20 μg/mL), or vehicle (DMSO), for 1 or 24 hours. Total RNA and protein were isolated.

Plasma analyses

Plasma estradiol and progesterone were quantified using ELISA kits from Calbiotech (Spring Valley, CA) and Genway (San Diego, CA), respectively. Triglycerides and cholesterol were quantified using an enzymatic colorimetric assay from Pointe Scientific (Canton, MI).

RNA isolation and quantitative PCR

Total RNAs were extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA), and complementary DNA (cDNA) was generated using High Capacity cDNA Synthesis (Applied Biosystems, Foster City, CA). RNA purity and concentration were assessed

using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Rockford, IL). mRNA expression was quantified by real time-qPCR using SYBR Green-based method (Applied Biosystems) for detection of amplified products. qPCR was performed in a 384-well plate format using the ViiA7 Real Time PCR machine (Life Technologies, Grand Island, NY). Ct values were converted to delta delta Ct values by adjusting to a reference gene (ribosomal protein l13a, Rpl13a) (Livak and Schmittgen, 2001). Primer sequences are listed in Table 3.2.

Western blot analysis

For animal tissues, livers and intestines were homogenized in sucrose-Tris buffer (pH=7.4-7.5) using the TissueLyser LT Adapter (Qiagen), per the manufacturer's protocol. Primary mouse hepatocytes were collected in PBS and cell pellets were resuspended in lysis buffer with 1% proteinase inhibitor. Protein concentrations were determined by BCA assay (Pierce Biotechnology, Rockford, IL). Fifty micrograms of liver or intestine homogenate or 30 micrograms of cell lysate protein were loaded onto a SDS-PAGE gel (4-12%, Life Technologies). Semi-quantification of expression was determined using primary antibodies raised against Fgf15 (Sc-27177, 1:1000, Santa Cruz), Ntcp (1:5000), Bsep (1:5000), phosphor-Erk1/2 (9101, 1:1000, Cell Signaling Technology, Danvers, MA) and Erk1/2 (9102, 1:1000, Cell Signaling Technology) followed by incubation with appropriate secondary antibody. Primary antibodies for Ntcp and Bsep were supplied by Dr. Bruno Stieger (University Hospital, Zurich, Switzerland). The intensity of band luminescence was acquired using a FluorChem E System Imager (ProteinSimple, Santa Clara, CA). β-Actin (ab8227, Abcam, Cambridge, MA) or Gapdh (ab2302, Abcam) were used as loading controls.

Indirect immunofluorescence

Liver cryosections (6 μm) were fixed in 4% paraformaldehyde for 5 minutes. Sections were blocked with 5% goat serum/PBS with 0.1% Triton X-100 (PBS-Tx) for 1 hour and then incubated with a primary antibody against Bsep diluted 1:100 in 5% goat serum, PBS-Tx for 2 hours at room temperature. Sections were then washed and incubated with an anti-rabbit secondary antibody linked to AlexaFluor488 (Life Technologies). Images were acquired on a Zeiss Observer D1 microscope at 20x using a Jenoptik camera. All sections were stained and imaged under uniform conditions for each antibody. Negative controls without primary antibody were also included (data not shown).

Plasma bile acid profiling

Plasma bile acid extracts were analyzed by a Thermo Accela Ultra Performance Liquid Chromatography system (Thermo Fisher Scientific) coupled with a Thermo LTQ XL Ion Trap Mass Spectrometer (ITMS, Thermo Fisher Scientific). Ionization was accomplished using Electrospray and the ITMS was operated in MS/MS mode with Selective Ion Monitoring. Simultaneous determination of 23 bile acids using commercial standards (Sigma-Aldrich and Steraloids, Newport, RI), was as previously reported (Zhan et al., 2016). The limit of quantification for all bile acids was 3.11 ng/μL plasma.

Statistical analysis

Data are presented as mean ± SE (animal studies) or mean ± SD (hepatocyte studies). Statistical analysis was performed using GraphPad Prism v6 (La Jolla, CA). mRNA and protein expression were analyzed by a 2-way ANOVA or a 1-way ANOVA followed by a Newman-Keul's multiple comparison post-hoc test when appropriate, to compare overall mean differences between groups. Significance was set at $p \leq 0.05$.

3.4 Results

Pregnancy outcomes following GW4064 treatment.

To determine the effects of Fxr activation on pregnancy indicators, plasma sex hormone concentrations and other indicators of physiological/pathological changes were quantified in pregnant mice treated with GW4064 (Table 3.1). As expected, plasma estradiol and progesterone levels increased by 1.5- and 6.5-fold with pregnancy, respectively. Progesterone levels were reduced by 20% with GW4064 treatment (20.3 ng/mL) compared to vehicle-treated pregnant mice (28.0 ng/mL), though still within the normal range for gestation day 14. Pregnancy increased plasma triglycerides by nearly 2-fold, which was not altered by treatment with GW4064. No significant differences were observed in plasma cholesterol levels among treatment groups on gestation day 14. The number of resorption sites at the time of sacrifice was similar between vehicle- and GW4064-treated mice. All fetuses appeared grossly normal. Additionally, both vehicle- and GW4064-treated pregnant mice had increased liver-to-body weight ratios as compared to virgin control mice.

Expression of hepatic bile acid-related genes in virgin and pregnant mice treated with GW4064.

Pregnancy and GW4064 treatment had no effect on Fxr mRNA levels (Fig. 3.1). Vehicle-treated pregnant mice exhibited a 55% reduction in Shp mRNA, as well as 35 to 40% decreased expression of Fgfr4, Cyp27a1, Bal and Baat mRNAs compared to vehicle-treated virgin mice. A 2-fold increase in Cyp7a1 expression, and a 55% elevation in Cyp8b1, was also observed in pregnant mice. Treatment of pregnant mice with GW4064 restored mRNAs of Shp, Cyp7a1, Cyp27a1 and Bal to virgin control levels. Down-regulation of Ntcp (53%) and Mrp3 (82%) transporter transcripts was also observed on gestation day 14 compared to virgin controls (Fig. 3.2A). A 40% decrease in Bsep

mRNA of vehicle-treated pregnant mice was noted but not statistically significant when compared to virgin controls. Treatment of pregnant mice with GW4064 up-regulated (25 to 150%) the expression of all hepatic transporters tested. Whereas GW4064 enhanced Ntcp, Bsep, Mrp3 and Ostβ expression in pregnant mice, little to no change in transporter expression was observed in virgin mice treated with GW4064.

At the protein level, down-regulation of Ntcp and Bsep by 50 and 38%, respectively, was confirmed in pregnant vehicle-treated mice (Fig. 3.2B). GW4064 treatment of pregnant mice moderately increased the expression of both Ntcp (15%) and Bsep (17%) proteins. Additionally, indirect immunofluorescent staining confirmed localization of Bsep to the canaliculi (Fig. 3.2C). Compared to vehicle-treated virgin mice, pregnancy reduced the intensity of Bsep staining which was restored by GW4064 treatment.

Expression of intestinal bile acid-related genes in virgin and pregnant mice treated with GW4064.

Decreased mRNAs of Fgf15, Shp and I-babp by 65%, 95% and 50%, respectively, were noted on gestation day 14 in vehicle-treated pregnant mice (Fig. 3.3A). While treatment of virgin mice with GW4064 nearly doubled Fgf15 expression, even greater induction was observed in pregnant mice (9-fold compared to vehicle-treated pregnant mice). GW4064 treatment also increased Ostα and Ostβ mRNA by 67% and 85% compared to virgin controls, respectively. Although I-babp mRNA levels returned to those seen in virgin controls following GW4064 treatment of pregnant mice, these data were not statistically significant. Neither pregnancy nor GW4064 treatment altered expression of Fxr or Asbt transporter mRNAs. Interestingly, Shp mRNA was nearly undetectable in vehicle-treated pregnant mice, but enhanced by 3.3-fold in GW4064-treated pregnant

mice as compared to vehicle-treated virgin mice. Similarly, Fgf15 protein was only detectable in pregnant mice following GW4064 treatment (Fig. 3.3B).

Bile acid pool analysis of plasma from virgin and pregnant mice treated with GW4064.

Overall, a trend for increased total bile acids was observed for vehicle-treated pregnant mice as compared to vehicle-treated virgin mice (Fig. 3.4A). This corresponded with a relative increase (100%) in the ratio of taurine:glycine conjugated bile acids. We further analyzed the bile acid pool composition by comparing relative percentages of individual bile acids (Fig. 3.4B). Of the bile acids that are most highly concentrated in the plasma of mice, a trend for reduced taurocholic acid (TCA) was observed in pregnant mice with GW4064 treatment compared to vehicle treatment (Fig. 3.4C). Short-term treatment of pregnant mice with GW4064 increased the relative percentage of the secondary bile acid ω-murcholic acid (ωMCA) compared to vehicle-treated virgin mice (18%) and compared to vehicle-treated pregnant mice (13%). Though deoxycholic acid (DCA) concentrations in the plasma were low in comparison to other bile acids, levels were significantly increased in GW4064-treated pregnant mice as compared to vehicle-treated virgin and pregnant mice (nearly doubled).

Modulation of Cyp7a1 expression by pregnant plasma in primary mouse hepatocytes.

To investigate the hypothesis that circulating factors present in blood lead to pregnancy-related bile acid changes, female naïve primary mouse hepatocytes were treated with 20% plasma isolated from virgin or pregnant (gestation day 17 or greater) mice. Treatment with plasma had no effect on hepatocyte cell viability as evidenced by negligible lactate dehydrogenase leakage (data not shown).

Subsequent studies tested whether the Fxr agonist GW4064 or recombinant FGF19 protein (the human ortholog of mouse Fgf15) could rescue pregnancy-related changes in bile acid synthesis pathways. For this effort, primary mouse hepatocytes were concomitantly treated with 20% plasma from virgin and pregnant mice and GW4064 or exogenous recombinant FGF19 protein. Activity of recombinant FGF19 protein was confirmed by increased phosphorylation of Erk1/2 protein after 1 hour of treatment (Fig. 3.5A) (Kong et al., 2012; Kong and Guo, 2014). Cyp7a1 mRNA expression was induced by 2.7-fold in the presence of pregnant plasma as compared to hepatocytes exposed to virgin plasma for 24 hours (Fig. 3.5B). Co-treatment of hepatocytes with pregnant plasma and either GW4064 or FGF19 reduced Cyp7a1 mRNA to levels at or below controls. For example, co-treatment with pregnant serum and recombinant FGF19 protein was able to reduce Cyp7a1 mRNA by 70 to 75%.

3.5 Discussion

The current study assessed the ability of activated Fxr to modulate hepatic and intestinal regulation of bile acid synthesis and transport pathways during pregnancy. As previously reported, pro-cholestatic adaptive changes in hepatic gene expression have been observed in pregnant mice (Milona et al., 2010a; Aleksunes et al., 2012; Song et al., 2014) in the absence of changes in the mRNA expression of Fxr itself (Fig.3.1). This included the repression of gene expression of Shp, Fgfr4, Cyp27a1, Bal, Baat, Ntcp, and Mrp3, and induction of the gene expression of Cyp7a1 on gestation day 14. This was further reflected in decreased protein levels of major basolateral and canalicular bile acid transporters, Ntcp and Bsep (Aleksunes et al., 2012). These data point to a functional impairment of Fxr activity during pregnancy more than a change in its expression. The coordinated down-regulation of sinusoidal uptake and efflux, as well as canalicular efflux transporters suggests that there is reduced bile acid enterohepatic recirculation, which may contribute to increased plasma bile acid levels late in pregnancy. A trend towards increased total bile acids in plasma was already apparent on gestation day 14 (Fig.3.4). Interestingly, treatment of pregnant mice with a specific Fxr agonist GW4064 restored the expression of bile acid synthesis enzymes and transporters towards levels typically observed in virgin mice with similar plasma hormone and biochemistry levels, resorption sites and viable fetuses among pregnant treatment groups.

It has been demonstrated in the rat that Fxr indirectly suppresses Ntcp expression through the inhibitory transcription factor Shp (Denson et al., 2001). Namely, activation of Fxr in the rat liver induces expression of Shp, which in turn inhibits the gene expression of Ntcp, a bile acid uptake transporter in hepatic sinusoidal membranes (Denson et al., 2001). However, analysis of the same Shp response element in human and mouse suggests this may not be the dominant or singular pathway for bile acid

regulation of NTCP/Ntcp in other species (Jung et al., 2004). Previously published literature confirms that during pregnancy, both Shp and Ntcp gene expression are concomitantly reduced (Milona et al., 2010a; Aleksunes et al., 2012). In this study, activation of Fxr in pregnant mice with the agonist GW4064 similarly induced both Shp and Ntcp (Fig.3.1 and 3.2). This co-regulation phenomenon for Shp and Ntcp has also been noted in newborn mice with the bile acid surge that initiates Fxr signaling. At birth, a positive correlation between Fxr and Ntcp mRNA is observed (Cui et al., 2012). Though not well-explored, Fxr response elements in the 5' promoter region of the Ntcp/*Slc10a1* gene have been identified in adult mice (Thomas et al., 2010). Therefore, a secondary mechanism for Fxr regulation of the bile acid uptake transporter Ntcp may occur in certain physiological states such as pregnancy.

GW4064 was identified and characterized as a potent, nonsteroidal and selective Fxr agonist in 2000 (Maloney et al., 2000). Pharmacokinetic studies in rats at the time of discovery revealed a poor oral bioavailability of 10% and a half-life of 3.5 hours (Maloney et al., 2000). GW4064 is commonly utilized as a pharmacological activator of Fxr in mice. It has been shown to decrease bile acid synthesizing enzymes Cyp7a1 and Cyp8b1 mRNA levels in an Fxr dependent manner, and not in Fxr-null mice (Kong et al., 2012). The ability of GW4064 to reduce expression of Cyp7a1 and Cyp8b1 and induce Shp and Fgf15 mRNA is mitigated by deletion of Fxr in all tissues of mice (Moschetta et al., 2004; Kong et al., 2012). Interestingly, more pronounced increases in intestinal and hepatic mRNA expression of Fxr targets were consistently observed following GW4064 treatment in pregnant mice as compared to virgin mice. Despite the low oral bioavailability of GW4064, it is possible that absorption of this lipophilic chemical is enhanced during pregnancy. This may result from reduced gastrointestinal motility in mice during pregnancy (Datta et al., 1974), similar to humans, which could lead to

greater residence time in the intestinal lumen and subsequent absorption into the body. Future studies utilizing long-term treatment of mice with GW4064 could help elucidate this mechanism, in addition to a clearer understanding of the ability of increased Fxr activation in altering bile acid pool composition.

The current study highlights the relationship between the restoration of hepatic Fxr target genes and direct activation of intestinal Fxr signaling by GW4064. Intestinal Fgf15 was inducible by 9-fold with GW4064 treatment during pregnancy (Fig. 3.3). This newly identified responsiveness in pregnant mice likely led to the attenuation of Cyp7a1 mRNA induction in the liver (Fig. 3.1). Further, in an isolated system where only primary mouse hepatocytes were exposed to pregnant plasma in the media, Cyp7a1 mRNAs were induced (Fig. 3.5). Similar to *in vivo* results it was observed that this enhanced expression could be abolished by not only a pharmacological Fxr agonist, but also the addition of FGF19, a positively regulated signaling molecule produced by activation of ileal Fxr. The lack of a sensitive method has precluded our ability to quantify Fgf15 protein levels in serum. Nonetheless, this study supports the novel hypothesis that intestinal bile acid pathways can be altered in pregnancy in an Fxr-dependent manner. Further, it is likely that Fxr in the intestine contributes to changes in hepatic gene expression due to the Fgf15/FGF19-mediated communication between the two organs as part of the coordinated regulation of bile acid homeostasis.

Sandwich-cultured fresh primary hepatocytes were utilized in this study because they behave similarly to hepatocytes *in vivo* and form bile canaliculi in culture to better mimic the liver and bile acid pathway. However, fresh mouse primary hepatocytes are not an ideal system to assess transporter expression and activity, which decrease over time in culture. This was evidenced by higher Ct values of hepatic transporters in primary

hepatocytes as compared to Ct values in mice (data not shown). Therefore, mRNA expression of uptake and efflux transporters measured in the primary mouse hepatocytes showed limited correlation to data from intact animals.

Data presented in this chapter suggest that activation of Fxr, most likely in the intestine due to the poor bioavailability of GW4064, can restore bile acid-related gene expression both in the intestine and the liver. Potential mechanisms for the restoration of liver bile acid pathways may occur through 1) direct activation of hepatic Fxr or 2) suppression of Cyp7a1 by up-regulation of Fgf15 levels. These may be important findings for the treatment of pregnancy-specific cholestatic liver disease. Pharmacological activation of Fxr in rodents prevents or resolves both intra- and extra-hepatic cholestasis (Liu et al., 2003; Moschetta et al., 2004). Other studies have shown that constitutively active Fxr in the intestine can reduce total bile acids and decrease bile acid pool hydrophobicity, and presumably, toxicity (Modica et al., 2012). Intestinal activation of Fxr specifically attenuated liver injury in models of extrahepatic cholestasis (bile duct ligation), as well as intrahepatic cholestasis (treatment with α-naphthylisothiocyanate and genetically induced). In this study, FGF19 treatment abrogated the pregnancy-related increase in Cyp7a1 expression *ex vivo*. Additional reports in the literature have confirmed that administration of FGF19 not only reduced CYP7A1 activity in healthy human patients but also protected mice from hepatotoxicity due to bile duct ligation and α-naphthylisothiocyanate treatment (Luo et al., 2014). The present study implicates not only hepatic, but for the first time, intestinal Fxr activation as a potential mechanism to regulate bile acid signaling during pregnancy and may represent a new therapeutic target for treating ICP.

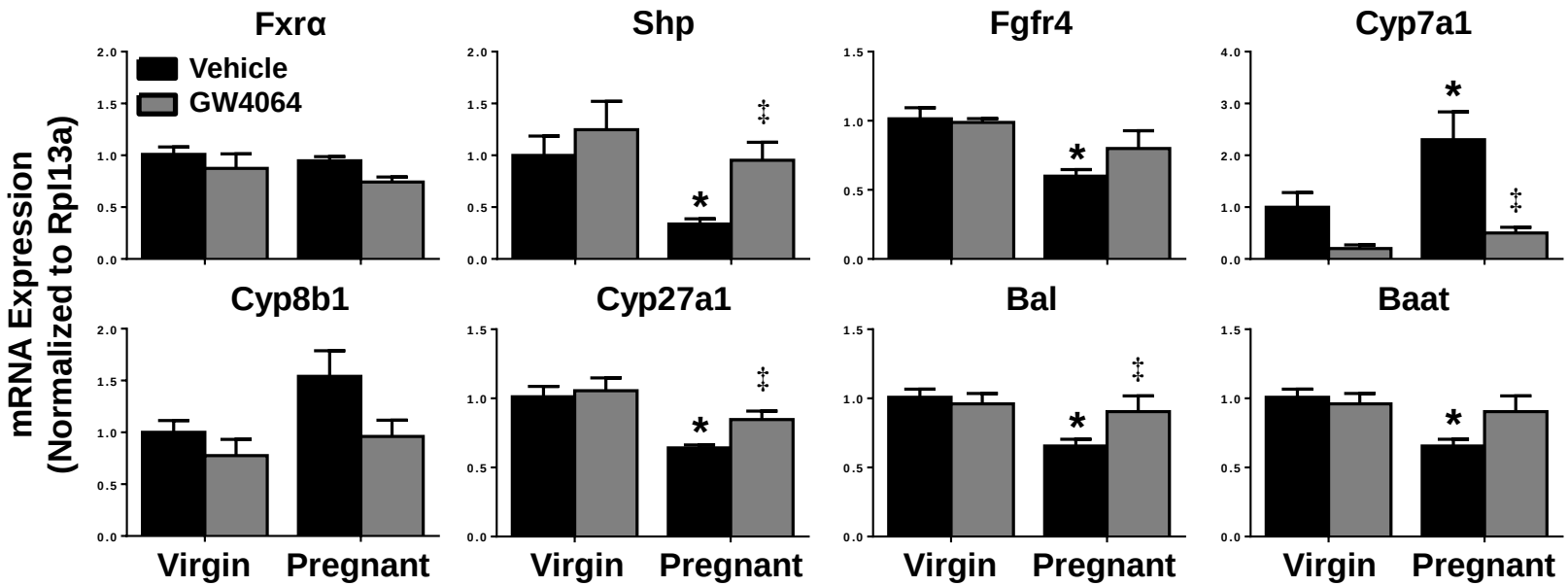

Fxrα
Shp
Fgfr4
Cyp7a1
Cyp8b1
Cyp27a1
Bal
Baat
Vehicle
GW4064
mRNA Expression
(Normalized to Rpl13a)
Virgin
Pregnant
*
‡

Fig. 3.1. Hepatic mRNA expression in vehicle- or GW4064-treated pregnant mice. Hepatic mRNA expression of Fxr signaling and bile acid synthesis and metabolic enzyme genes was quantified in vehicle- and GW4064-treated virgin and pregnant mice on gestation day 14. Data were normalized to vehicle-treated virgin controls (set to 1.0) and presented as mean relative expression ± SE (n=5-6). Black bars represent vehicle-treated mice and grey bars represent GW4064-treated mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with virgin mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

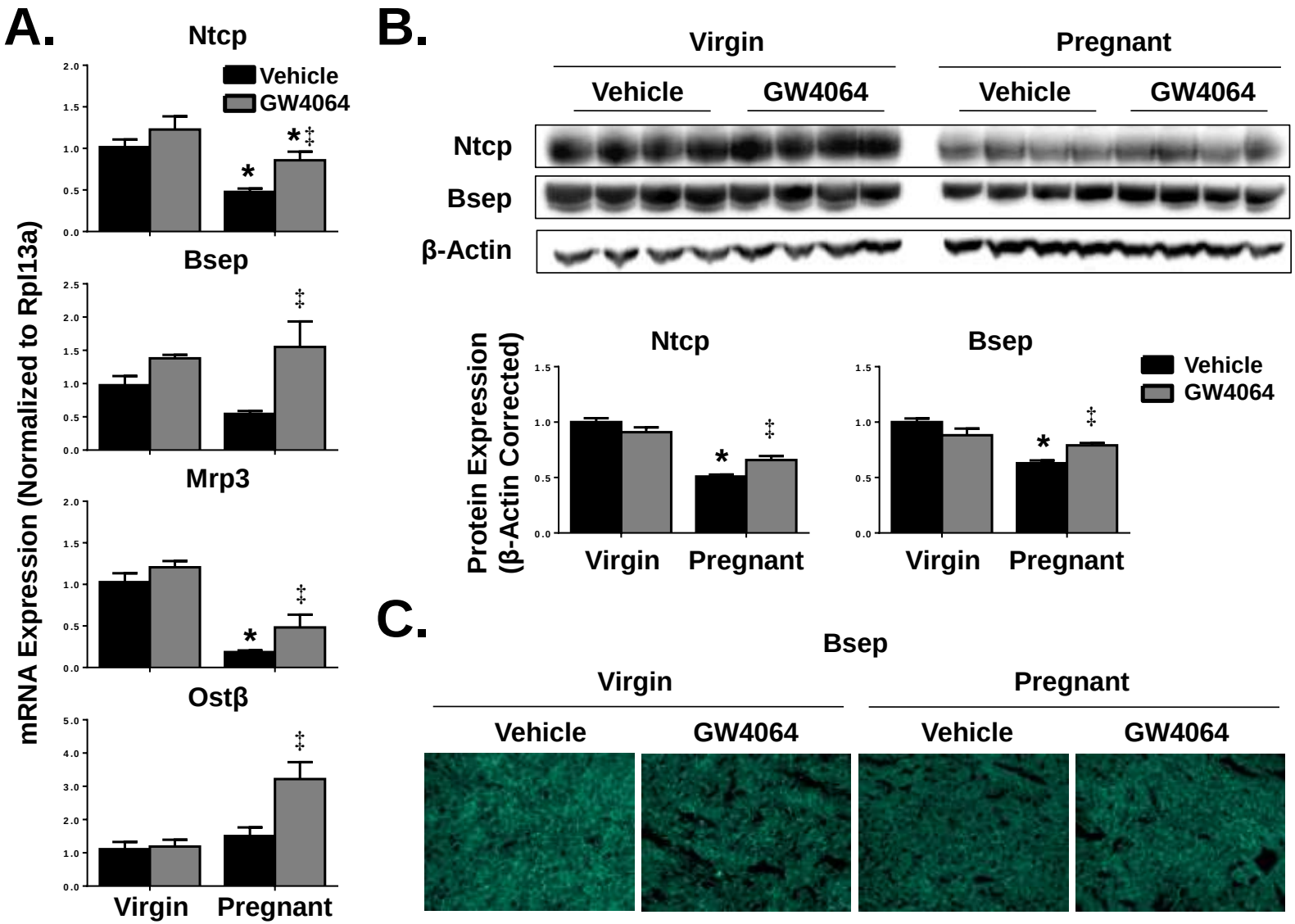
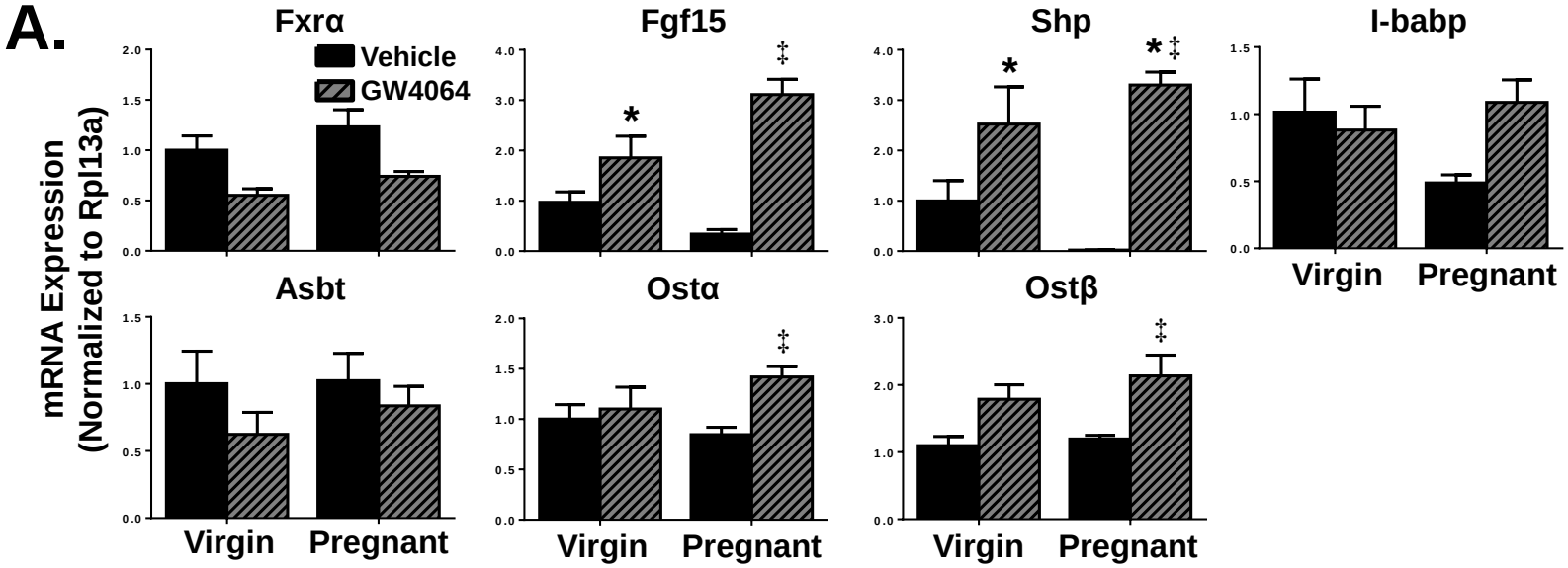

A.
Ntcp
Vehicle
GW4064
Bsep
Mrp3
Ostβ
mRNA Expression (Normalized to Rpl13a)
Virgin
Pregnant
B.
Virgin
Pregnant
Vehicle
GW4064
Ntcp
Bsep
β-Actin
Protein Expression (β-Actin Corrected)
C.
Bsep

Fig. 3.2. Hepatic transporter expression in vehicle- or GW4064-treated pregnant mice. Hepatic (A) mRNA and (B) protein expression of uptake and efflux transporters were quantified in vehicle- and GW4064-treated virgin and pregnant mice on gestation day 14. Western blots were performed using whole liver homogenates, and protein band intensity was semi-quantified. β-Actin was used as a loading control. Data were normalized to vehicle-treated virgin controls (set to 1.0) and presented as mean relative expression ± SE (n=5-6). Black bars represent vehicle-treated mice and grey bars represent GW4064-treated mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with virgin mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated pregnant mice. (C) Indirect immunofluorescence against canalicular transporter Bsep (*green*) was conducted on liver cryosections (6 μm). Representative images are shown. Magnification x100.


A.
Fxrα
Fgf15
Shp
I-babp
Vehicle
GW4064
mRNA Expression
(Normalized to Rpl13a)
Asbt
Ostα
Ostβ
Virgin
Pregnant
*
‡

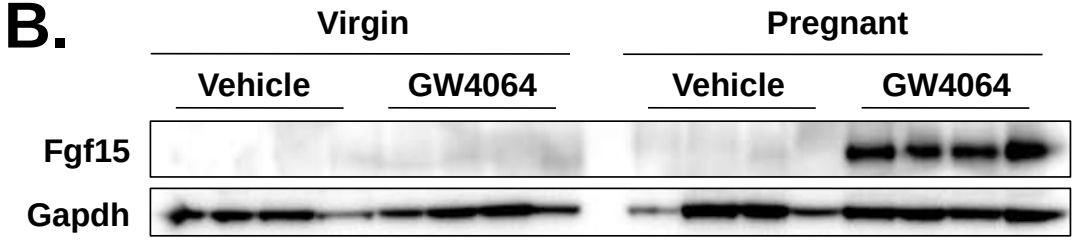

B.
Virgin
Pregnant
Vehicle
GW4064
Fgf15
Gapdh

Fig. 3.3. Ileal gene and protein expression in vehicle- or GW4064-treated pregnant mice. (A) mRNA and (B) protein expression of Fxr-regulated targets were quantified in ilea from vehicle- and GW4064-treated virgin and pregnant mice on gestation day 14. Western blot staining of Fgf15 protein was performed using whole ileal homogenates. Data were normalized to vehicle-treated virgin controls (set to 1.0) and presented as mean relative expression ± SE (n=5-6). Black bars represent vehicle-treated mice and grey diagonal striped bars represent GW4064-treated mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated virgin mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

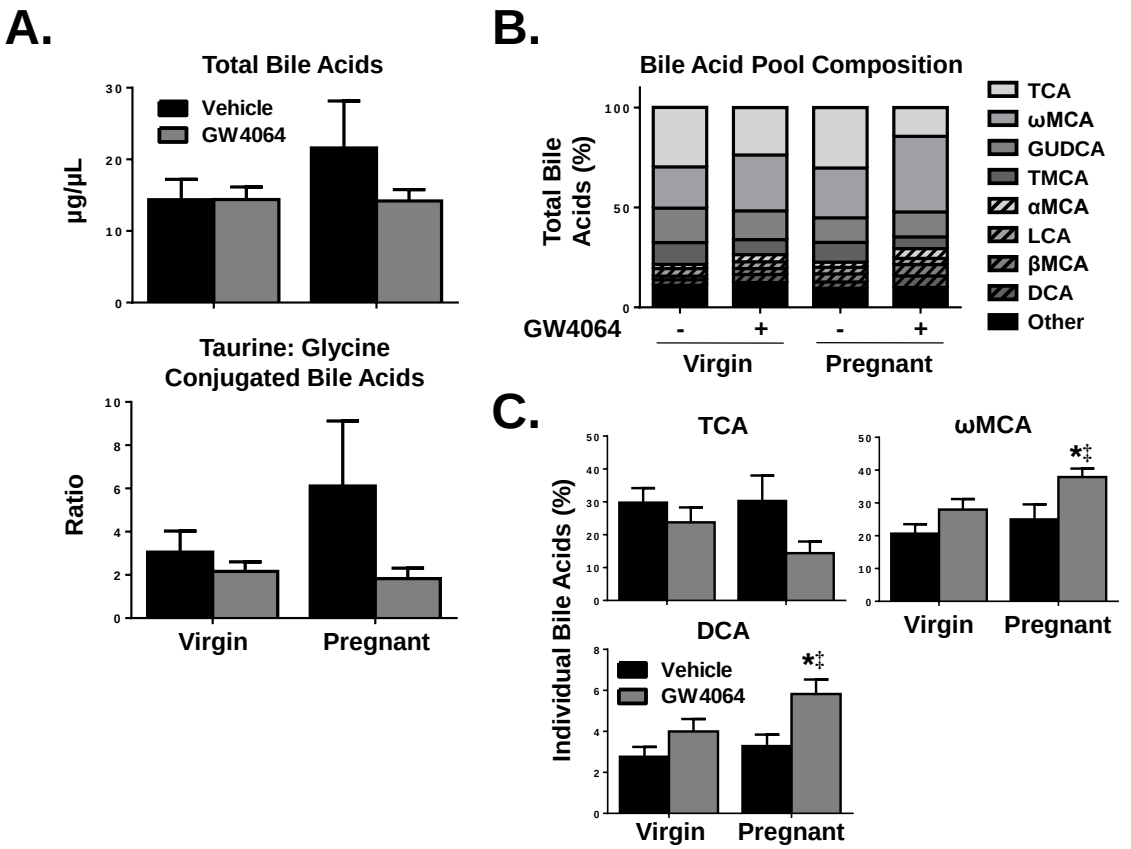


Fig. 3.4. Plasma bile acid profiling of virgin and pregnant mice after short term treatment with GW4064. (A) Total and (B, C) individual bile acids were determined in vehicle- and GW4064-treated virgin and pregnant mice on gestation day 14. Individual bile acids are shown as a percentage of total bile acids per group. Data are presented as mean relative expression ± SE (n=5). Black bars represent vehicle-treated mice and grey bars represent GW4064-treated mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated virgin mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

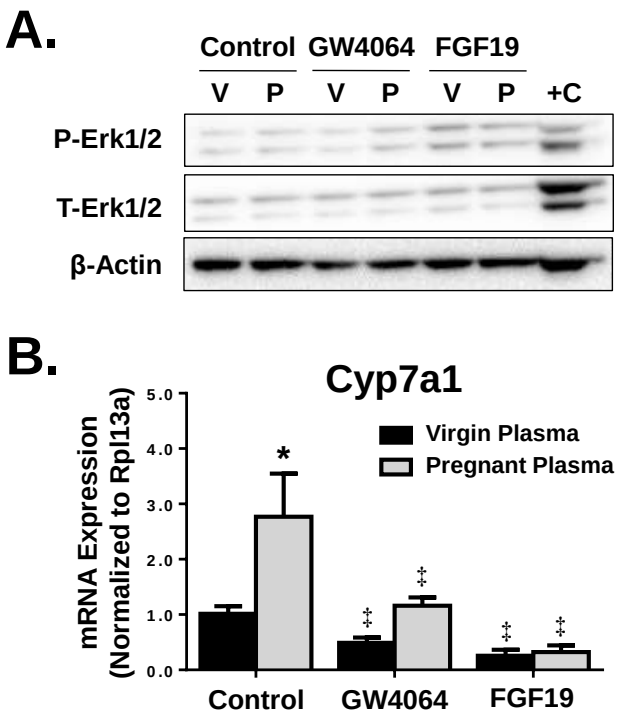


Fig. 3.5. Cyp7a1 regulation in primary mouse hepatocytes. (A) Erk1/2 activation as indicated by phospho-Erk1/2 (P-Erk) compared to total Erk1/2 (T-Erk) after 1 hour treatment and (B) mRNA and protein expression of Cyp7a1 after 24 hour treatment of primary mouse hepatocytes with media containing 20% pooled virgin or pregnant mouse plasma, in the presence of GW4064 (5 μM), recombinant FGF19 protein (20 μg/ml), or vehicle (DMSO). Western blots were performed using whole cell lysates. V, virgin plasma; P, pregnant plasma, mouse liver positive control. Data are presented as mean relative expression ± SD (n=3). Black bars represent virgin mouse plasma-treated hepatocytes and grey bars represent pregnant mouse plasma-treated hepatocytes. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with plasma-treated virgin controls. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with plasma-treated pregnant controls.

Table 3.1. Pregnancy endpoints following GW4064 treatment[1].

	Virgin Vehicle	Virgin GW4064	Pregnant Vehicle	Pregnant GW4064
Liver: Body weight	0.0569±0.0019	0.0554±0.0017	0.0647±0.00070*	0.0681±0.0010*
Resorptions	N/A	N/A	1.00±0.36	0.667±0.33
Estradiol (pg/mL)	7.91±0.4	7.85±1.3	12.8±0.8*	12.1±2.0
Progesterone (ng/mL)	3.70±1.0	2.68±0.6	28.0±1.8*	20.3±3.2*‡
Triglycerides (mg/dL)	36.9±1.1	36.1±2.7	65.0±4.2*	55.7±4.7*
Cholesterol (mg/dL)	91.0±5.7	100.7±3.1	90.9±3.6	92.3±3.1

[1] Body weights, liver weights and number of resorptions were recorded at the time of sacrifice. Total litter sizes ranged from 1 to 9 (mean 5.5). Circulating progesterone, estradiol, total bile acids, triglycerides and cholesterol were quantified for vehicle- and GW4064-treated mice on gestation day 14. Data are presented as mean ± SE (n=5-6). Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated virgin mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with vehicle-treated pregnant mice. N/A: not applicable.

Table 3.2. qPCR primer sequences

Primer	Forward (5’ to 3’)	Reverse (5’ to 3’)
Asbt	TTGCACAGCACAAGCAGTGA	TGCATTGAAGTTGCTCTCAGGT
Baat	CCTGAGACATCCTAAGGTCCT	TACTGGGTACAGGTGGGTAGA
Bal	TCCCAAAGCCAGCCATCTTA	GTGGCTCCAACTTGTAAGCAG
Bsep	TAGACAGGCAACCCGTCATGGAC	ATCCCACGAACGCCGTCGTT
Cyp7a1	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA
Cyp8b1	AGTACACATGGACCCCGACATC	GGGTGCCATCCGGGTTGAG
Cyp27a1	GCCTCACCTATGGGATCTTCA	TCAAAGCCTGACGCAGATG
Fgf15	GCCATCAAGGACGTCAGCA	CTTCCTCCGAGTAGCGAATCAG
Fgfr4	CTTTGGGCAAGTGGTTCGTG	GAGACCAGGTCTGCCAAATC
Fxrα	TCCGGACATTCAACCATCAC	TCACTGCACATCCCAGATCTC
I-babp	CCCCAACTATCACCAGACTTC	ACATCCCCGATGGTGGAGAT
Mrp3	CTGGGTCCCCTGCATCTAC	GCCGTCTTGAGCCTGGATAAC
Ntcp	ACGTCCTCAAGGCAGGCATGAT	AGCCCATCAGGAAGCCAGTGAAAG
Ostα	CACTGGCTCAGTTGCCATTT	GCATACGGCATAAAACGAGGT
Ostβ	GCAAACAGAAATCGAAAGAAGC	TCTGGCAGAAAGACAAGTGAT
Rpl13a	CAAGAAAAAGCGGATGGTGG	TCCGTAACCTCAAGATCTGC
Shp	CGATCCTCTTCAACCCAGATG	AGGGCTCCAAGACTTCACACA

CHAPTER 4: PREGNANCY-RELATED CHANGES IN LIVERS AND ILEA OF MICE LACKING THE FARNESOID X RECEPTOR

Jamie E Moscovitz[a], Bo Kong[a], Grace L Guo[a,b], Lauren M Aleksunes[a,b]

[a] Department of Pharmacology and Toxicology, Rutgers University Ernest Mario School of Pharmacy, 170 Frelinghuysen Rd., Piscataway, NJ 08854, USA

[b] Environmental and Occupational Health Sciences Institute, 170 Frelinghuysen Rd., Piscataway, NJ 08854, USA

4.1 Abstract

Bile acid homeostasis is often dysregulated in pregnancy and can present as asymptomatic hypercholemia to the more severe disease, intrahepatic cholestasis of pregnancy (ICP). Recent studies in humans and mice suggest that women with ICP have enhanced susceptibility to future hepatobiliary disease, while their offspring are at risk for respiratory distress and mortality. It has been proposed that pregnancy represents a state of Farnesoid X Receptor (human FXR/mouse Fxr) inactivation, with hepatic gene changes in pregnant mice that mirror those of naïve Fxr knockout mice. The objective of this study was to determine the degree to which Fxr is necessary for the adaptive hepatic and intestinal gene responses of pregnant mice. For this purpose, livers and intestines were collected from virgin and pregnant wild-type and Fxr-null mice on gestation day 17. Regardless of pregnancy status, ilea of Fxr-null mice exhibited reduced expression of Fxr target genes (Fgf15, Shp, I-babp, Asbt, Ostβ) when compared to wild-type mice. Expression of ileal Fgf15, Shp and I-babp, as well as hepatic Ostβ and Bsep, was down-regulated in pregnant wild-type, but not in Fxr-null mice. Gene expression analysis of the hepatic transcription factors AhR, Car, Pxr and Ppar and their prototypical target genes demonstrate that virgin Fxr-null mice have increased expression of these pathways. However, AhR, Car and Pxr are repressed in pregnant Fxr-null mice as compared to virgin Fxr-null mice. Data from this study demonstrate that Fxr deficiency prevents the repression of key bile acid regulatory factors and transporters in the intestines, and to a lesser extent, in the livers of pregnant dams.

Abbreviations

AhR, aryl hydrocarbon receptor; Asbt, apical sodium-dependent bile acid transporter; Bsep, bile salt export pump; Car, constitutive androstane receptor; Cyp, cytochrome P450; Fgf, fibroblast growth factor; Fxr, farnesoid X receptor; I-babp, ileal bile acid binding protein; ICP, intrahepatic cholestasis of pregnancy; Mdr, multidrug resistance protein; Mrp, multidrug resistance-associated protein; Ntcp, Na+-taurocholate cotransporting polypeptide; Ost, organic solute transporter; Ppar, peroxisome proliferator-activated receptor; Pxr, pregnane X receptor; Shp, small heterodimer partner

4.2 Introduction

Pregnancy can increase circulating bile acid levels leading to asymptomatic hypercholemia (40% of pregnancies) or the more severe disease, intrahepatic cholestasis of pregnancy (ICP, 0.5-2% of pregnancies). ICP enhances the transfer of bile acids from mother to fetus, which increases the risk of fetal morbidity and mortality due to preterm labor, fetal distress and intrauterine death (Tan, 2003; Abu-Hayyeh et al., 2013b). In addition, recent research in humans and mice suggests that women with ICP have greater susceptibility to future hepatobiliary disease, while offspring born to mothers with ICP are at higher risk of developing metabolic disorders (Marschall et al., 2013; Papacleovoulou et al., 2013). While the mechanisms by which pregnancy leads to cholestatic disease are still being explored, altered hepatic transport of biliary constituents and xenobiotics has emerged as a potential contributing factor (Dixon et al., 2000; Dixon et al., 2009; Aleksunes et al., 2012).

The Farnesoid X Receptor (Fxr) is a transcription factor that is activated in the presence of bile acids to promote their enterohepatic recirculation. In the liver and intestine, Fxr cooperatively regulates the expression of transcription factors (including the small heterodimer partner, Shp, in the liver) and endocrine factors (fibroblast growth factor, Fgf15, in the intestine) to suppress bile acid synthesis enzymes of the classic pathway, including cytochrome P450s (Cyp) 7a1 and 8b1. Fxr also regulates the transcription of hepatic and intestinal uptake and efflux transporters involved in the disposition of bile acids and other bile constituents. Prior research has proposed that pregnancy represents a “state of Fxr inactivation,” with hepatic gene changes in pregnant mice that mirror those of naïve Fxr knockout mice (Milona et al., 2010a; Aleksunes et al., 2012). Accordingly, the hepatic and intestinal mRNA expression of Fxr in pregnant mice is

similar to virgin mice (Milona et al., 2010a; Aleksunes et al., 2012), suggesting that the reduced functioning of Fxr is due to post-transcriptional regulation. High circulating concentrations of steroid hormones, specifically estrogen and progesterone metabolites, have been proposed as functional inhibitors of Fxr signaling (Vallejo et al., 2006; Milona et al., 2010a; Aleksunes et al., 2012; Abu-Hayyeh et al., 2013a; Song et al., 2014; Chen et al., 2015).

Previous studies indicate that mRNA of intestinal Fgf15, as well as hepatic Shp, Cyp7a1 and Cyp8b1 are greatly impacted by the deletion of Fxr from the respective tissues, as well as the whole body (Kim et al., 2007a; Kong et al., 2012). For example, deletion of Fxr in the liver, intestine, or both tissues increases Cyp8b1 mRNA in liver, while Cyp7a1 mRNA is elevated by loss of Fxr in the intestine. Further, Fxr deficiency has been associated with enhanced susceptibility to cholestasis, gallstone disease, nonalcoholic steatohepatitis, and spontaneous formation of liver and colon tumors in mice (Kim et al., 2007b; Yang et al., 2007; Kong et al., 2009; Maran et al., 2009). Genetic variants of FXR that alter its function, and loss-of-function variants of target transporters BSEP and MDR3 have been implicated in the etiology of ICP in humans (Dixon et al., 2000; Van Mil et al., 2007; Dixon et al., 2009; Dixon et al., 2014). Our hypothesis is that loss of hepatic and intestinal Fxr signaling during pregnancy primes susceptible women to developing ICP by reducing the expression of bile acid transporters and enhancing bile acid synthetic enzymes. The objective of this study was to determine whether altered expression of bile acid enzymes and hepatobiliary transporters during pregnancy involve changes in Fxr signaling using mice lacking Fxr.

4.3 Materials and methods

Animals

Adult male and female wild-type C57BL/6 mice (8-12 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited animal care facility in temperature-, light- and humidity-controlled rooms. Studies were approved by the Rutgers University Institutional Animal Care and Use Committee, and were in accordance with national guidelines. Fxr-null mice were maintained on a pure C57BL/6 genetic background (Sinal et al., 2000; Guo et al., 2006). A subset of female wild-type and Fxr-null mice were mated overnight with male mice of the same genotype and checked for the presence of a vaginal sperm plug (designated gestation day 0) the next day. The remaining female mice were used as virgin controls. Mice had access to standard chow and water *ad libitum*. Liver and intestines were collected from time-matched virgin and pregnant wild-type and Fxr-null mice (n=6-7) on gestation day 17. Small intestines were divided into three equal segments, representing the duodenum, jejunum and ileum. Ileal fragments were utilized for all studies. Liver and total body weights were recorded for female mice at the time of sacrifice. Tissues were collected within a 60-minute period in the morning (Zhang et al., 2011b), snap frozen in liquid nitrogen, and stored at -80°C.

RNA isolation and quantitative PCR

Total RNAs were extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA), and complementary DNA (cDNA) was generated using High Capacity cDNA Synthesis (Applied Biosystems, Foster City, CA). RNA purity and concentration were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Rockford, IL). Messenger RNA expression was quantified by real time-qPCR using the SYBR Green-

based method (Applied Biosystems) for detection of amplified products. qPCR was performed in a 384-well plate format using the ViiA7 Real Time PCR machine (Life Technologies, Grand Island, NY). Ct values were converted to delta delta Ct values by adjusting to a reference gene (ribosomal protein l13a, Rpl13a) (Livak and Schmittgen, 2001). Specific forward and reverse primer sequences are listed in Table 4.1.

Western blot analysis

Livers were homogenized in sucrose-Tris buffer (pH=7.4-7.5) using the TissueLyser LT Adapter (Qiagen), per the manufacturer's protocol. Protein concentrations were determined by BCA assay (Pierce Biotechnology, Rockford, IL). Fifty micrograms of protein was loaded onto a SDS-PAGE gel (4-12%, Life Technologies). Semi-quantification of transporter expression was determined using primary antibodies raised against Ntcp (1:5000, K4, Dr. Bruno Stieger), and Bsep (1:5000, K44, Dr. Bruno Stieger) followed by incubation with an anti-rabbit secondary antibody (1:1000 Ntcp, 1:2000 Bsep). The intensity of band luminescence was acquired using a FluorChem E System Imager (ProteinSimple, Santa Clara, CA). β-Actin (1:2000, ab8227, Abcam, Cambridge, MA) was used as a loading control.

Total bile acid quantification

Total serum and hepatic bile acids were quantified using an enzymatic colorimetric assay (Genzyme Diagnostics, San Diego, CA). For hepatic bile acids, whole liver homogenates were made with the TissueLyser LT Adapter (Qiagen, Valencia, CA), per the manufacturer's protocol. Hepatic bile acids were protected from light and extracted at room temperature overnight in 1 mL of tert-butanol: water (1:1), centrifuged at 10,000 x g (4°C) and assayed immediately.

Statistical analysis

Data are presented as mean ± SE. Statistical analysis was performed using GraphPad Prism v6 (La Jolla, CA). mRNA and protein expression were analyzed by a 2-way ANOVA or a 1-way ANOVA followed by a Bonferroni or Newman-Keul's multiple comparison post-hoc test when appropriate, to compare overall mean differences between groups. Significance was set at $p \leq 0.05$.

4.4 Results

Expression of intestinal bile acid homeostasis genes in pregnant Fxr-null mice.

Messenger RNA expression of intestinal Fxr was not altered by pregnancy in wild-type mice and was not detected in Fxr-null mice (Fig. 4.6). The basal expression of ileal Fxrα, the endocrine growth factor Fgf15, transcription factor Shp, bile acid binding protein I-babp, and the transporters Asbt and Ostβ mRNAs in virgin Fxr-null mice was markedly reduced by 40 to 99% compared to virgin wild-type controls (Fig. 4.1). Compared to time-matched virgin control mice, mRNA levels of Fgf15, Shp, I-babp and Ostα were reduced by 55 to 80% in the ileum of pregnant wild-type mice. Pregnancy did not alter the expression of the bile acid transporters Asbt and Ostβ in wild-type mice. Unlike wild-type mice, pregnancy did not further alter Fgf15, Shp or I-babp mRNA levels in Fxr-null mice.

Expression of hepatic genes involved in bile acid homeostasis in pregnant Fxr-null mice.

As observed in the intestines, null mice did not express Fxr mRNA in the liver (Fig. 4.6). Similar to a published report (Milona et al., 2010a), virgin mice lacking Fxr had reduced basal mRNA expression of the hepatic bile acid transcription factor, Shp (50%), and the bile acid transporters Ntcp (23%), Bsep (62%), and Ostβ (98%), relative to virgin wild-type mice (Fig. 4.2). Conversely, virgin Fxr-null mice exhibited enhanced mRNA expression of bile acid synthesis gene Cyp7a1 (3-fold), as well as the Mrp3 transporter (47%), relative to virgin wild-type mice.

Hepatic Bsep and Ostβ mRNAs were decreased 35 to 60% in pregnant wild-type dams, but not in pregnant Fxr-null mice as compared to their respective virgin controls. Pregnancy reduced the gene expression of Shp, Ntcp and Mrp3 in the livers of wild-type mice as well as Fxr-null mice (Fig. 4.2). Interestingly, Cyp7a1 expression in pregnant

Fxr-null mice was enhanced 5-fold compared to wild-type controls. Alternatively, pregnancy in Fxr-null mice did not further increase Cyp8b1 mRNA (data not shown). Western blot analysis of the critical bile acid uptake and canalicular efflux transporters Ntcp and Bsep, respectively, was performed in virgin and pregnant wild-type and Fxr-null mice (Fig. 4.3A). Semiquantitation of band intensities confirmed the decrease of both Ntcp and Bsep proteins in pregnant wild-type and Fxr-null mice (Fig. 4.3B).

Hepatic transcription factor signaling in pregnant Fxr-null mice.

Additional studies were performed to understand novel mechanisms that mediate pregnancy-related changes in bile acid and transport pathways that are independent from Fxr. The aryl hydrocarbon receptor (AhR) and its prototypical target gene Cyp1a1 mRNAs were increased between 1.5- and 2-fold in virgin Fxr-null mice, and pregnancy in this genotype prevented the up-regulation of Cyp1a1 gene expression (Fig. 4.4). Similarly, prototypical target genes of both the constitutive androstane receptor (Car) and pregnane X receptor (Pxr), namely Cyp2b10 and Cyp3a11, respectively, were up-regulated 3-fold in virgin Fxr-null mice. Pregnancy reduced their expression back to virgin wild-type levels. Conversely, basal expression of a classical peroxisome proliferative-activator receptor (Ppar) alpha target gene, Cyp4a14, was reduced 55% in Fxr-null mice. Pregnancy down-regulated its expression by 80-85% in both genotypes. No differences in Lxr, Srebp-1, Erα or Hnf1α and Hnf4α mRNA expression were observed in pregnant Fxr-null mice (data not shown). Taken together, wild-type and Fxr-null mice differentially express hepatic regulatory transcription factors and their prototypical target genes, however pregnancy similarly represses their expression.

Gross endpoints and bile acid levels in pregnant Fxr-null mice.

Quantification of total bile acids indicated that both virgin and pregnant Fxr-null mice had doubled the hepatic bile acid levels of wild-type counterparts (Fig. 4.5). No differences were noted between pregnant mice and virgin controls. The induction in hepatic bile acids was complimented by a modest increase in liver weights of Fxr-null mice, as indicated by elevated liver:body weight ratios. Alternatively, no differences were observed in plasma total bile acid concentrations across genotypes. In a second set of mice, an increase in bile acid levels did not occur until gestation day 19 (data not shown). While pregnant wild-type and Fxr-null mice had a similar number of resorptions, litter sizes were reduced in Fxr-null mice (6.33 ± 0.21) compared to wild-type mice (8.66 ± 0.42).

4.5 Discussion

The current study investigated the intestinal and hepatic Fxr regulation of bile acid synthesis and transport pathways during pregnancy using Fxr-null mice. As expected, mice lacking Fxr had lower basal expression of intestinal and hepatic genes that are critically involved in bile acid homeostasis. Pregnancy-related changes in bile acid pathways, notably the down-regulation of Fgf15 gene expression, were largely absent in the intestines of Fxr-null mice. However, down-regulation of a few key bile acid transporters still occurred in pregnant mice in the absence of constitutive Fxr expression. The absence of Fxr and its role in altered hepatic bile acid homeostasis of pregnancy is complicated by unique expression profiles of critical transcription factors among Fxr-null and wild-type mice that may compensate for loss of a critical signaling pathway.

Given the prior assertion that liver gene expression in pregnancy represents a state of Fxr inactivation (Milona et al., 2010a), it was anticipated that Fxr-null mice would not exhibit the adaptive gene changes that are typically observed in wild-type mice during pregnancy. This hypothesis was largely confirmed in the intestines of Fxr-null mice, which had markedly reduced constitutive expression of targets that were not altered by pregnancy. The absence of Fgf15, Shp, I-babp and significant reduction of Asbt and Ostβ basal expression in Fxr-null mice confirms that genes involved in intestinal bile acid regulation are almost exclusively transcriptionally regulated by Fxr. However, partial suppression of some hepatic Fxr target genes including Shp, Ntcp and Mrp3 was still observed in pregnant Fxr-null mice. To identify alternate transcription factors that might regulate these genes in the absence of Fxr, the mRNA expression of multiple nuclear receptors and transcription factors, as well as their respective prototypical target genes was analyzed in the livers of Fxr-null mice (Fig. 4.4). The attenuation and down-regulation of these pathways in Fxr-null mice by pregnancy, particularly of Car and Pxr,

could account for the observed differences in Mrp3 expression. These signaling pathways should be investigated further to describe alterations to xenobiotic disposition genes in Fxr-null mice. Interestingly, we have previously shown that treatment of wild-type mice with TCPOBOP, a specific Car agonist, was not as effective at inducing Mrp3 in pregnant mice as it was in virgin mice (Bright et al., 2016).

Pregnancy is a unique physiological state in which Shp and Ntcp expression are coordinately down-regulated (Milona et al., 2010a; Aleksunes et al., 2012), whereas their relationship is typically inverse in rodent models of cholestasis (Trauner et al., 1998a; Sinal et al., 2000; Fickert et al., 2001). Another example of the coordinated regulation of Shp and Ntcp occurs during the bile acid surge at birth (day 0) in mice (Cui et al., 2012). Namely, expression of bile acid and Fxr target genes is up-regulated at postnatal day 0 in pups. Surprisingly, the further suppression of Shp in pregnant Fxr-null mice was not a result of induction of liver X receptor (Lxr) and the positive feedback mechanisms (second to Fxr negative feedback) associated with bile acid homeostasis (data not shown). It has been demonstrated that Shp can be influenced by circadian rhythm in lactating rats at various hours of post-partum day 10 compared to virgin controls (Wooton-Kee et al., 2010). In addition, the circadian regulation of Shp has been identified in livers of male mice (Oiwa et al., 2007). Utilizing an electrophoretic mobility shift assay, the authors demonstrated that CLOCK-BMAL1 protein, a mediator of circadian rhythm, could directly bind the mouse *Nr0b2/Shp* promoter in the immortalized CV-I kidney and HepG2 hepatoma cell lines (Oiwa et al., 2007). Future studies may explore whether Fxr-null mice are more sensitive to regulators of circadian rhythm, or if ‘clock genes’ are alternatively expressed in livers of these mice.

The relationship between proper Fxr functions and liver cholesterol, triglyceride and bile acid homeostasis is well-documented. Though this study suggests that Fxr may not be singularly responsible for pro-cholestatic gene changes in pregnancy, work from our laboratory has demonstrated that activation of Fxr with a pharmacological agonist in pregnant mice can resolve the dysregulation of bile acid homeostasis (Chapter 3). It is possible that there are differences in the bile acid pool composition among virgin and pregnant, wild-type and Fxr-null mice. Not only Fxr signaling, but the quantity of conjugated versus unconjugated bile acids may modulate bile acid transporter gene and protein expression. Genome-wide intestine and liver binding sites of Fxr have been characterized in mice with chromatin immunoprecipitation (ChIP) coupled with massively parallel sequencing in mice (Thomas et al., 2010). Binding of Fxr to the promoter regions of target genes in virgin and pregnant animals could be assessed by ChIP-qPCR to determine whether Fxr binding is reduced in pregnant mice. Future studies may also determine the specific roles of hepatic versus intestinal Fxr in adaptive changes seen in pregnancy with tissue-specific knockout mice.

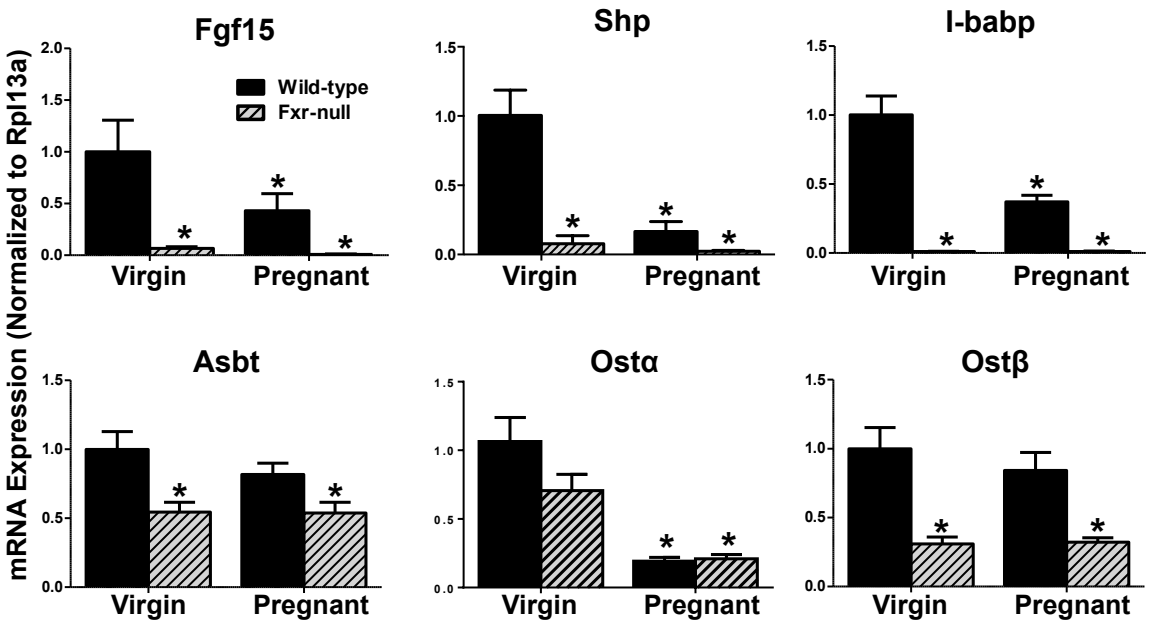


Fig. 4.1. Ileal mRNA expression in pregnant Fxr-null mice. mRNA expression of Fxr-regulated ileal genes were quantified in virgin and pregnant wild-type and Fxr-null mice on gestation day 17. Data were normalized to virgin, wild-type controls (set to 1.0) and presented as mean relative expression ± SE (n=6-7). Black bars represent wild-type mice and grey bars represent Fxr-null mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with virgin wild-type mice.

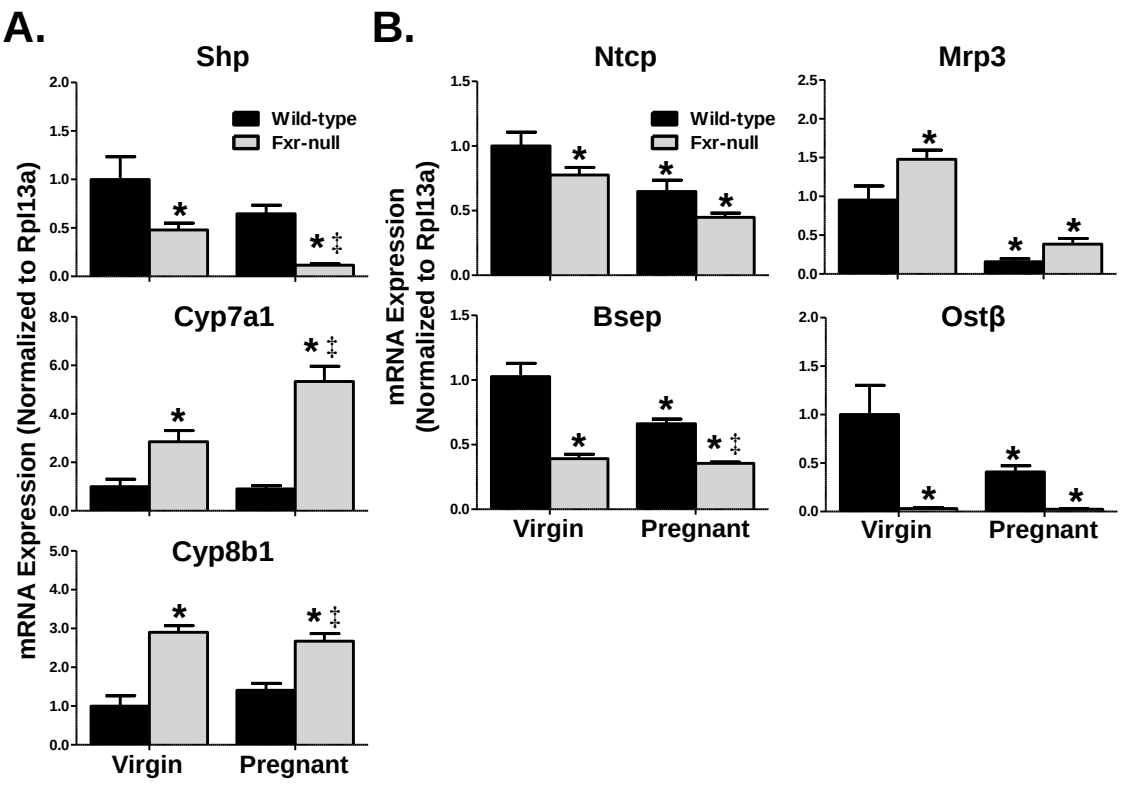


Fig. 4.2. Hepatic mRNA expression in pregnant Fxr-null mice. Hepatic mRNA expression of (A) transcription factor and bile acid synthesis and (B) uptake and efflux transporter genes were quantified in virgin and pregnant wild-type and Fxr-null mice on gestation day 17. Data were normalized to virgin, wild-type controls (set to 1.0) and presented as mean relative expression ± SE (n=6-7). Black bars represent wild-type mice and grey bars represent Fxr-null mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with virgin wild-type mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with pregnant wild-type mice.

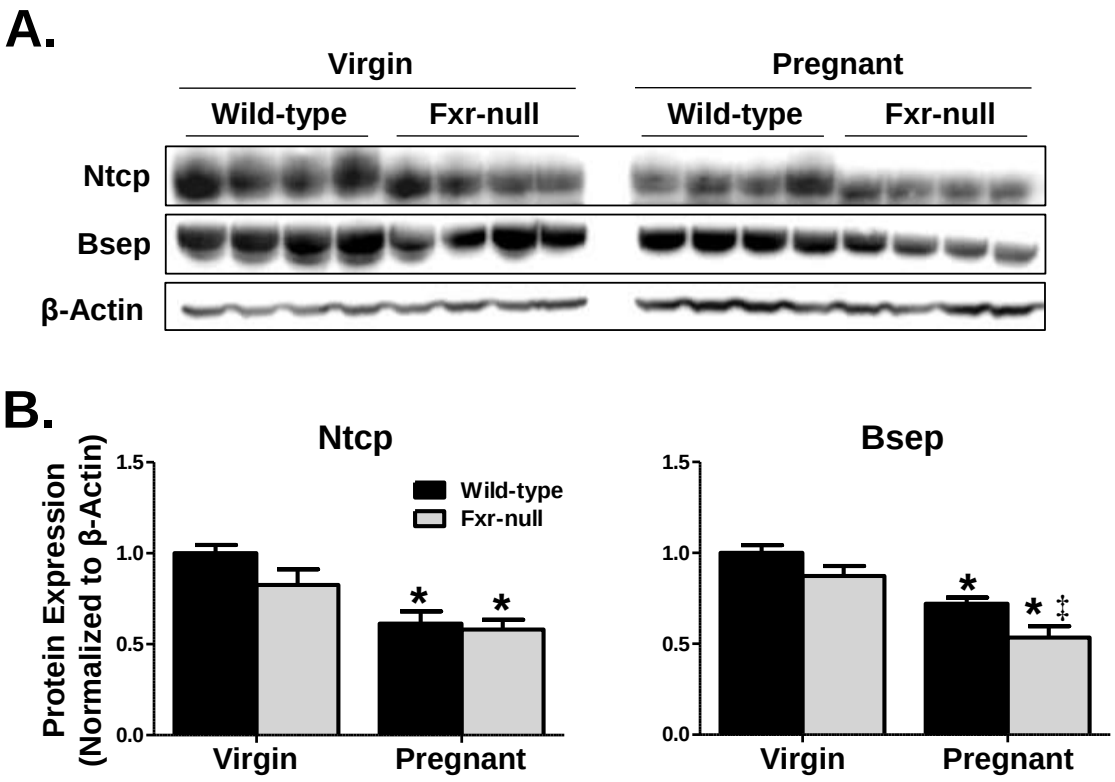


Fig. 4.3. Hepatic expression of bile acid transporter proteins in pregnant Fxr-null mice. (A) Western blots were performed using whole liver homogenates from virgin and pregnant wild-type and Fxr-null mice on gestation day 17 (B) Protein band intensity was semi-quantified from two independent experiments. Data were normalized to virgin wild-type controls (set to 1.0) and presented as mean relative expression ± SE (n=6-7). Black bars represent wild-type mice and grey bars represent Fxr-null mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with virgin wild-type mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with pregnant wild-type mice.

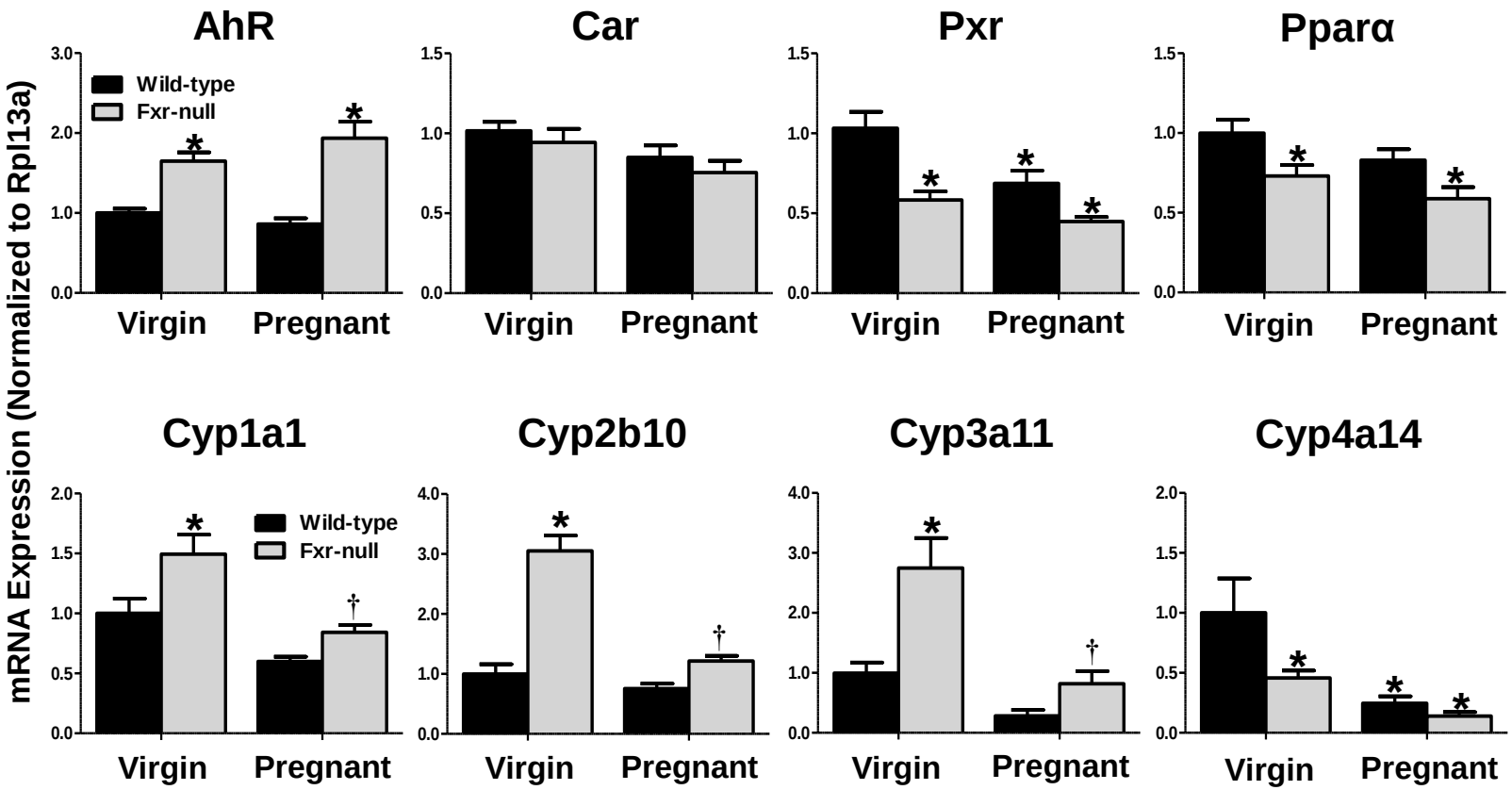

AhR
Car
Pxr
Pparα
Cyp1a1
Cyp2b10
Cyp3a11
Cyp4a14
mRNA Expression (Normalized to Rpl13a)
Wild-type
Fxr-null
Virgin
Pregnant
*
†

Fig. 4.4. Hepatic mRNA expression of transcription factor-related pathways in virgin and pregnant Fxr-null mice. Hepatic mRNA expression of (A) transcription factors and (B) prototypical target genes was quantified in virgin and pregnant wild-type and Fxr-null mice on gestation day 17. Data were normalized to virgin wild-type controls (set to 1.0) and presented as mean relative expression ± SE (n=6-7). Black bars represent wild-type mice and grey bars represent Fxr-null mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with virgin wild-type mice. Daggers (†) represent statistically significant differences ($p \leq 0.05$) compared with virgin Fxr-null mice.

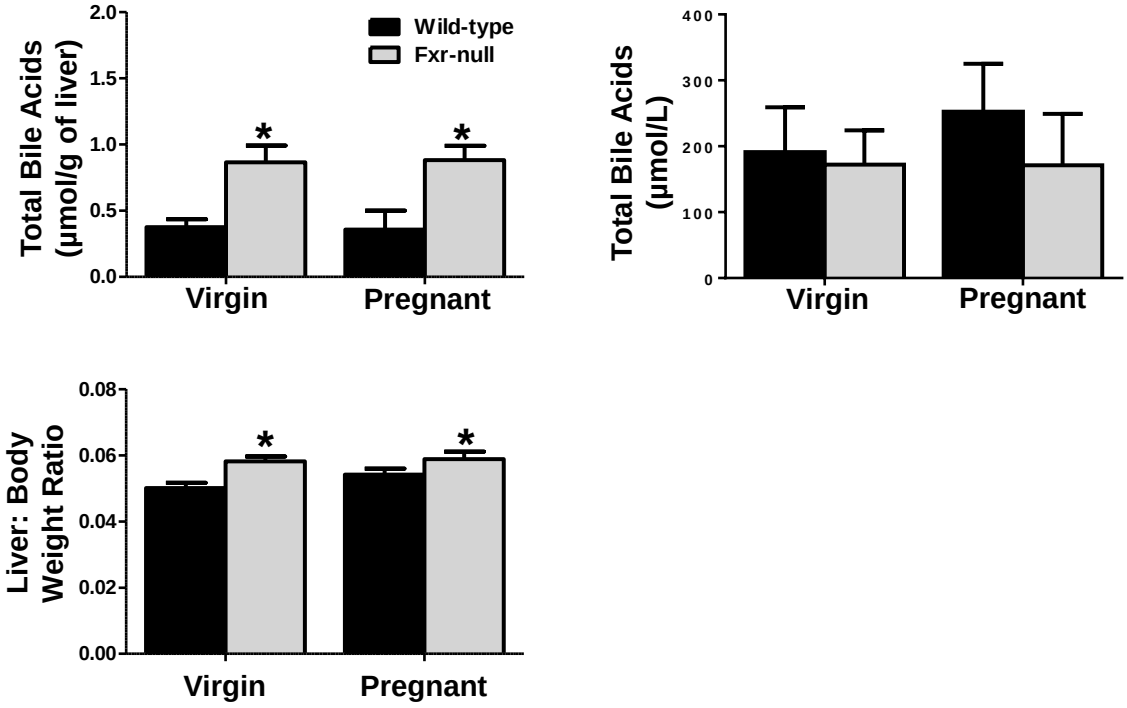


Fig. 4.5. Total bile acid levels and liver to body weight ratios in virgin and pregnant Fxr-null mice. Levels of total serum and hepatic bile acids, as well as liver: body weight ratios for time-matched virgin and pregnant Fxr-null mice on day 17. Black bars represent wild-type mice and grey bars represent Fxr-null mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with virgin wild-type mice.

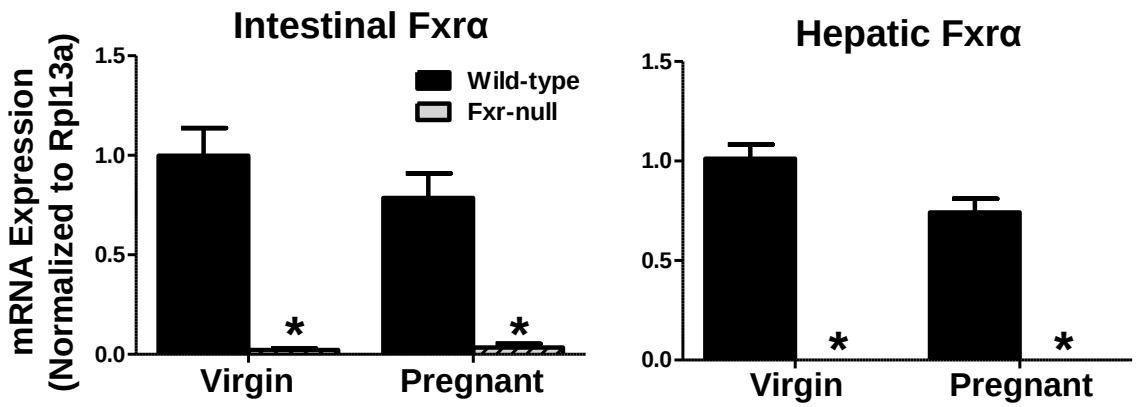


Fig. 4.6. Fxrα mRNA expression in pregnant Fxr-null mice. mRNA expression of intestinal and hepatic Fxr was quantified in virgin and pregnant wild-type and Fxr-null mice on gestation day 17. Data were normalized to virgin, wild-type controls (set to 1.0) and presented as mean relative expression ± SE (n=6-7). Black bars represent wild-type mice and grey bars represent Fxr-null mice. Asterisks (*) represent statistically significant difference ($p \leq 0.05$) compared with virgin wild-type mice. Double daggers (‡) represent statistically significant difference ($p \leq 0.05$) compared with pregnant wild-type mice.

Table 4.1. qPCR primer sequences

Primer	Forward (5’ to 3’)	Reverse (5’ to 3’)
AhR	GGAGCGCTGCTTCCTCCACA	AGCTGCCCTTTGGCATCACAAC
Asbt	TTGCACAGCACAAGCAGTGA	TGCATTGAAGTTGCTCTCAGGT
Bsep	TAGACAGGCAACCCGTCATGGAC	ATCCCACGAACGCCGTCGTT
Car	TCCAGGAGCCTGAGTATGTG	ACTTTGGAGCCGAGACTGTT
Cyp1a1	AAGTGCAGATGCGGTCTTCT	AAAGTAGGAGGCAGGCACAA
Cyp2b10	TGCTGTCGTTGAGCCAACCTTCA	GGGGCTCCCTGGGATTTCCG
Cyp3a11	CCCAGTGGGGATAATGAGTAA	CTTGCCTTTCTTTGCCTTCT
Cyp4a14	TTGCTCACGAGCACACAGATGGA	CCTCTGCACGCAGGTCCTCAT
Cyp7a1	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA
Cyp8b1	AGTACACATGGACCCCGACATC	GGGTGCCATCCGGGTTGAG
Fgf15	GCCATCAAGGACGTCAGCA	CTTCCTCCGAGTAGCGAATCAG
Fxrα	TCCGGACATTCAACCATCAC	TCACTGCACATCCCAGATCTC
I-babp	CCCCAACTATCACCAGACTTC	ACATCCCCGATGGTGGAGAT
Mrp3	CTGGGTCCCCTGCATCTAC	GCCGTCTTGAGCCTGGATAAC
Ntcp	ACGTCCTCAAGGCAGGCATGAT	AGCCCATCAGGAAGCCAGTGAAAG
Ostβ	GCAAACAGAAATCGAAAGAAGC	TCTGGCAGAAAGACAAGTGAT
Pparα	GCGTGGTGCATTTGGGCGTA	GGCCACAGAGCGCTAAGCTGT
Pxr	GCAGCATCTGGAACTACCAA	CTGGTCCTCAATAGGCAGGT
Rpl13a	CAAGAAAAAGCGGATGGTGG	TCCGTAACCTCAAGATCTGC
Shp	CGATCCTCTTCAACCCAGATG	AGGGCTCCAAGACTTCACACA

CHAPTER 5: OVERALL DISCUSSION AND CONCLUSIONS

Summary

The overarching objective of this dissertation research was to characterize adaptations of the intestine and intestine-liver signaling that can contribute to altered chemical disposition, as well as sensitize some women to develop liver disease, during late pregnancy. It is reported that 64% of pregnant women take prescription medications (excluding prenatal vitamins) throughout pregnancy (Andrade et al., 2004). These drugs are predominantly administered by the oral route and therefore intestinal absorption is required to reach target sites of action. For this reason, a better understanding of the molecular and biochemical adaptations occurring in the intestines during pregnancy is warranted. Moreover, there is a need to characterize the pharmacological responses of the liver and intestines during pregnancy with the goal of identifying a potential therapeutic target to treat women with cholestatic liver disease.

Both *in vivo* and *in vitro* data presented in this dissertation support the hypothesis that third-trimester relevant concentrations of steroid and placental hormones can impair the signaling of nuclear receptor pathways (Pxr and Fxr) that are part of the intestine-liver axis and lead to dysregulation of xenobiotic and bile acid disposition pathways. Four aims tested this hypothesis: 1) Assess the mRNA and protein expression patterns of intestinal bile acid homeostasis and transport pathways in pregnant mice (**CHAPTERS 2 and 3**) 2) Determine whether steroid and placental hormones alter bile acid signaling and transport in the intestines (**CHAPTER 2**) 3) Determine the ability of Fxr activation to restore bile acid synthesis and transport expression in pregnant mice (**CHAPTER 3**) and 4) Assess the impact of loss of hepatic and intestinal Fxr signaling on pregnancy-induced bile acid changes (**CHAPTER 4**).

The first aim characterized the expression profiles of intestinal Fxr and Pxr target enzymes and transporters in mice during late pregnancy (gestation days 14, 17 and 19). For this and subsequent *in vivo* studies included in this dissertation, intestines were segmented at the time of collection into duodenum, jejunum and ileum, and ileal fragments were profiled for changes to mRNA and protein expression. The ileum is the most distal part of the small intestine, and is a site of active absorption in humans and rodents for both bile acids (Baker and Searle, 1960; Weiner and Lack, 1962; Krag and Phillips, 1974) and exogenous compounds (Artursson et al., 1993; Ungell et al., 1998). Specific Fxr target genes (Fgf15, I-babp and Shp) were down-regulated on gestation days 14 and 17 while the target transporter Ostα was reduced on gestation day 17 (Fig. 2.1 and 2.3). These findings suggest that expression of genes and proteins responsible for homeostasis of endogenous bile acids is altered in the small intestine during pregnancy, which is explored further in Chapters 3 and 4. Most importantly, an overall reduction in the mRNA and protein expression of the Pxr target, Cyp3a11, and apical efflux transporters (Mdr1, Mrp2 and Bcrp) was noted across gestational time points (Fig. 2.1 and 2.2). These data suggest that there is a reduction in expression of proteins responsible for the luminal efflux of xenobiotics, which may impact function.

The second aim examined the role of steroid and placental hormones in the down-regulation of FXR and PXR in the intestine. In the absence of a validated *in vitro* model of the small intestine, these experiments were conducted utilizing human LS174T colon adenocarcinoma cells transfected with the human *FXR* plasmid (LS174T-FXR) to more closely mimic the higher expression of FXR compared to PXR observed in the ileum as opposed to the colon. This aim also served a role in providing translational data for the observed changes in pregnant mice from Aim 1. LS174T-FXR cells were treated for 24 hours with third-trimester relevant concentrations of estradiol, progesterone,

epiallopregnanalone-sulfate (PM5S), placental lactogen, cortisol, growth hormone, testosterone and dehydroepiandrosterone. Treatment with progestins (progesterone and PM5S) most closely paralleled changes in efflux transporter expression observed in pregnant mice on gestation day 14 and 17 (Fig. 2.2 and Fig. 2.5). Namely, progestins reduced expression of the PXR target gene CYP3A4, as well as MDR1 and MRP2 in LS174T-FXR cells. Interestingly, progesterone induced BCRP mRNA. Similar to progestin treatment, placental lactogen treatment repressed MRP2 and induced BCRP, but also up-regulated MDR1 and attenuated MRP3 mRNA expression (Fig. 2.6). These data suggest a progestin-mediated reduction in PXR signaling evidenced by transporter repression.

The third and fourth aims assessed the hepatic and intestinal Fxr regulation of bile acid synthesis and transport pathways during pregnancy using complementary gain-of-function (Fxr agonist treatment) and loss-of-function (Fxr-null mice) approaches. Both animal studies confirmed the novel hypothesis that intestinal bile acid pathways can be altered in pregnancy in an Fxr-dependent manner. Further, it is likely that Fxr in the intestine contributes to changes in hepatic gene expression due to the Fgf15-mediated communication between the two organs as part of the coordinated regulation of bile acid homeostasis. These findings may be important for the treatment of pregnancy-specific cholestatic liver disease.

Intestinal Molecular Adaptations in Mouse Pregnancy

Similar to the global down-regulation of transporters in the livers of mice (Aleksunes et al., 2012), suppression of apical efflux transporters was observed in the ileum (Fig. 2.2). Mdr1, Mrp2 and Bcrp are responsible for the transport of diverse substrates. The Mrp2 and Bcrp transporters are tasked with removing endogenous substrates, such as

glucuronide- and sulfate-conjugated bilirubin, bile acids and steroid hormones (reviewed in Klaassen and Aleksunes, 2010). Reduced intestinal efflux and retention of endogenous molecules could represent an advantageous physiological adaptation during normal pregnancy, and contribute to the maintenance of high concentrations of these molecules during pregnancy. Some substrates of the intestinal apical efflux transporters, however, include drugs commonly prescribed to pregnant women such as the antiretroviral drugs ritonavir and nelfinivir, antidiabetic drugs such as glyburide, and antibiotics such as erythromycin and nitrofurantoin (Andrade et al., 2004; Klaassen and Aleksunes, 2010). A small cohort study conducted in 13 women at gestational weeks 28-32 and then 6-10 weeks post-partum described increased renal MDR1 activity during pregnancy as indicated by enhanced urine concentrations of midazolam and digoxin (Hebert et al., 2008). Interestingly, another study demonstrated a 70% decrease in systemic exposure of orally administered nitrofurantoin in pregnant Bcrp-null mice as compared to non-pregnant Bcrp-null mice, whereas this phenomenon was not observed in wild-type mice (Zhang et al., 2009). Authors suggest a compensatory mechanism of induction with other intestinal apical efflux transporters in Bcrp-null mice. Future studies could utilize oral administration of relevant drugs in wild-type, Mdr1-, Mrp2-, and Bcrp-null mice to determine differences in drug plasma concentrations and pharmacokinetic parameters during pregnancy across genotypes. These studies could delineate whether transporter function, in addition to expression, is compromised in pregnant mice, as well as the relative contributions of each transporter to overall functional changes. Given the global repression of ileal efflux transporters in pregnant mice, it may be beneficial to analyze the expression profiles of jejunal uptake transporters in late gestation, such as organic cation and organic anion transporters.

We have a limited understanding of how intestinal absorption of drugs might be altered during human pregnancy due to multiple factors. First, ethical concerns limit the testing of drugs in pregnant women. Second, determining the relative contribution of transport to altered drug disposition for drugs that are extensively metabolized would be difficult in this population. For the majority of drugs, pharmacokinetic data in pregnant women are generated post-marketing. Further, only women who are already prescribed a medication by their physician can be enrolled in a pharmacokinetic study (USFDA, 2004). The current paradigm of data collection for drug use in pregnant women relies heavily on physician reporting of adverse events, and a lengthy period of time to compile results in enough subjects to reform dosing regimens (reviewed in Anger and Piquette-Miller, 2008). Therefore, while caution should be taken in extrapolating expression data for transporters and across species, data presented in this dissertation could explain a possible mechanism for reduced apical efflux (leading to enhanced uptake) of oral drugs during pregnancy. The evidence of diminished intestinal efflux transporter expression in pregnancy may particularly need to be considered in combination with information regarding pregnant women with genetic variants of MDR1, MRP2 and BCRP, as well as women consuming dietary or chemical (drug) inhibitors of these transporters. For example, treatment of Caco-2 cells with the antidepressant fluoxetine (10 µM) resulted in 30% inhibition of MDR1 transport of radiolabeled digoxin (Pariante et al., 2003).

Steroid and Placental Hormone Regulation of Nuclear Receptors

A candidate hormone approach was employed to determine the potential contribution(s) of individual hormone treatments to bile acid and transporter regulation in LS174T-FXR cells. Not only are estradiol, progesterone, and PM5S elevated in healthy pregnant women, but further enhancement of steroid hormone concentrations were noted in women with ICP and oral contraceptive-induced cholestasis (Pasmant et al., 2012; Abu-

Hayyeh et al., 2013a; Chen et al., 2015; Abu-Hayyeh et al., 2016). While steroid hormones are elevated in both mouse and human pregnancy, the magnitude by which they increase varies. Additionally, time of peak concentrations may differ. Progesterone levels taper off in pregnant mice prior to parturition, while they remain high until term in humans (McCormack and Greenwald, 1974; Creasy and Resnik, 2009). Moreover, two variants of placental lactogen are produced in mice that peak in early (placental lactogen-I) or late (placental lactogen-II) gestation, whereas only one form steadily increases throughout pregnancy in humans (Soares et al., 1982; Ogren et al., 1989; Creasy and Resnik, 2009).

In vitro research conducted in Chapter 2 implicated progesterone and PM5S in down-regulation of PXR controlled transporters (Fig. 2.4). It also introduced a potential role for placental lactogen in the restoration of transporter expression prior to parturition (Fig. 2.6). MDR1 (LS174T-FXR cells) and Mdr1a (mouse ilea) were reduced by progestins and on gestation days 14-17, respectively. MDR1 was inducible by placental lactogen in LS174T-FXR cells and Mdr1a was restored on gestation day 19 to virgin control levels. Alternatively, MRP2 (LS174T-FXR cells) and Mrp2 (mouse ilea) were repressed by both progestins and placental lactogen in LS174T-FXR cells and remained low through gestation day 19, respectively. Though these experiments cannot be interpreted as a side-by-side comparison between species, these observations warrant further analysis of the role of placental lactogen in regulation of intestinal xenobiotic transporters, and its interaction with progesterone.

ABC-transporter function assays for MDR1, MRP and BCRP in the presence and absence of steroid and placental hormones would expand upon the gene profiling data included in this dissertation. An assay utilizing fluorescent cell imaging and transport of

rhodamine123, calcein AM and Hoechst 3342 to measure MDR1, MRP(1-3) and BCRP function, respectively, has been developed by our laboratory (Bircsak et al., 2013). Future studies should optimize the use of this technology with LS174T-FXR cells. For example, it is anticipated that treatment with progesterone would reduce MDR1 function in LS174T-FXR cells, thereby increasing intracellular concentrations of rhodamine123, while treatment with placental lactogen would enhance MDR1 function, and reduce intracellular concentrations of its substrate compared to controls.

Due to the identification of PXR as an orphan nuclear receptor activated by pregnanes (Kliewer et al., 1998; Synold et al., 2001), it is anticipated that PXR mediated the effects of progestins on down-regulation of CYP3A4 and target efflux transporter gene expression (Fig. 2.4 and 2.5). To determine if this effect could instead be mediated by progesterone activation of its cognate endocrine nuclear receptor, gene regulatory regions of CYP3A4, MDR1, MDR2 and BCRP were analyzed by the NUBIScan algorithm to predict possible progesterone receptor response elements (DR3s) (Podvinec et al., 2002). Genomic sequences for CYP3A4, MDR1, MDR2 and BCRP were acquired from the UCSC genome browser (Human December 2013 (GRCh38/hg38) assembly), and included the promoter regions (2000 bases) upstream of the target gene, and 1000 base pairs downstream. A custom matrix for the progesterone receptor response element was generated in NUBIscan. The two response element half sites AGAACA (sequence 1) and TGTTCT (sequence 2) were included with equal weight of importance (Ham et al., 1988). Query sequences for the upstream and downstream regions of each gene of interest were entered into NUBIScan for comparison with the custom matrix. An automatic search of predicted sites was conducted with a raw score threshold of 0.95. Results of this search for all four promoter

region and downstream sequences suggest that it is unlikely that progesterone receptor response elements are present.

Data generated in primary mouse hepatocytes indicated that the mixture of hormones present in pregnant plasma could induce the bile acid synthesis enzyme Cyp7a1 (Fig. 3.5). Primary hepatocytes grown on collagen with a matrigel overlay (i.e. sandwich-cultured) retain more *in vivo* characteristics of liver cells, including the ability to form functional canalicular networks. The sandwich-culturing model presents the opportunity to conduct a cross-species study including primary hepatocytes isolated from mice, rats and humans and treated with pregnant plasma isolated from each respective species. This study would allow for the comparison of the effects of pregnant serum on hepatic bile acid homeostasis genes in each species. Future studies should also assess the impact of the mixture of hormones that peak in the third trimester on expression profiles of ileal enzymes and transporters. This could be accomplished with *ex vivo* culture of ilea (Cima et al., 2004) from wild-type mice with the same 20% exposure to serum isolated from mice in late gestation. I hypothesize that the *ex vivo* treatment of ilea with pregnant plasma would recapitulate the suppression of the Fxr and Pxr target genes observed *in vivo*, implicating the combination of hormones in interference with nuclear receptor signaling.

Restoration of Fxr Function in Pregnancy

The goal of the studies performed in Chapters 3 and 4 was to provide proof-of-concept data demonstrating Fxr as a potential therapeutic target in the treatment of ICP. While total plasma bile acid and individual bile acid species concentrations in combination with gene and protein expression data can inform on the pro-cholestatic status of pregnancy, cholestasis is defined as a reduction in bile flow. Therefore, a next step in this study

would be the evaluation of bile flow rate in virgin and pregnant vehicle- and GW-treated mice. Further, these data would be complimented by an understanding of the interaction of Fxr and DNA-binding to target genes during pregnancy, in the absence and presence of GW4064. Fxr-DNA binding could be analyzed by chromatin immunoprecipitation coupled with qPCR, or electrophoretic mobility shift assay.

The alleviation of pruritus, the main symptom of ICP, has been studied independently of the progression of cholestasis itself. In 40 ICP patients UDCA treatment and resolution of pruritus were associated with a 55% decrease of pregnanediol disulfate metabolites in urine (Glantz et al., 2008). A correlation between improved pruritus scores and decreased disulphated progesterone metabolites was apparent after 3 weeks of UDCA treatment while no association with improved serum bile acid levels was observed (Glantz et al., 2008). More recently, scratching behavior was studied in wild-type and knockout mice of the G protein coupled receptor, Tgr5 (Abu-Hayyeh et al., 2016). Activated by bile acids, Tgr5 has previously been implicated in stimulation of release of itching-related neuropeptides (Alemi et al., 2013) and has been studied further in pruritis associated with sulfated progesterone metabolites and ICP. Scratching events in wild-type and Tgr5-null mice at the intradermal injection site of vehicle or PM3S were recorded over 60 minutes (Abu-Hayyeh et al., 2016). These studies showed that PM3S injection resulted in a 21-fold increase in scratching events compared to vehicle injection in wild-type mice, and this response was attenuated in Tgr5-null animals (Abu-Hayyeh et al., 2016). Though parallel and both implicating sulfated progesterone metabolites, it is likely that distinct pathways are regulating pruritus and increased serum bile acid levels in ICP. More studies are needed that analyze both pruritis and bile acid levels in ICP patients and models of maternal cholestasis to determine if there are additional unifying factors.

In order to determine whether pharmacological activation of Fxr can resolve gene changes in a disease model, future studies should make use of a recently published animal model of ICP. Not only do Bsep-null female mice present with increased serum bile acid levels and serum alkaline phosphatase, but pups exhibit the negative outcomes associated with ICP, including neonatal lethality from respiratory distress by 24 hours after birth (Zhang et al., 2012; Zhang et al., 2015). Moreover, Bsep-null mice demonstrate a reversal of the maternal-fetal bile acid gradient, similar to that observed in human ICP cases (Laatikainen, 1975; Zhang et al., 2015). Investigators demonstrated that crossing Bsep-null mice with Pxr-null mice reduced bile acid reuptake in the ileum, and decreased maternal serum bile acid levels and improved neonatal survival (Zhang et al., 2015). Determining whether activation of Fxr could overcome the maternal and postnatal phenotypes of Bsep-null mice would represent the next step in establishing Fxr as a therapeutic target to treat liver disease during pregnancy. For the proposed study, I would orally dose virgin and pregnant wild-type and Bsep-null mice with vehicle or GW4064 from gestation days 13 to 17 and assess total bile acid levels in maternal serum and fetuses, maternal serum ALT/AST/ALP, and the number of viable/nonviable fetuses on gestation day 18. I would expect both maternal and fetal total bile acids to be reduced in GW4064-treated pregnant Bsep-null mice as compared to vehicle-treated pregnant Bsep-null mice, and be similar values to those of wild-type controls. I would also anticipate that GW4064 treatment could reduce serum biomarkers of liver disease in pregnant Bsep-null mice. Finally, I would expect to observe that the treatment of pregnant Bsep-null mice with GW4064 would result in more viable fetuses than those treated with vehicle.

There are currently multiple clinical trials in progress for selective FXR agonists to treat primary biliary cholangitis, primary sclerosing cholangitis, fibrosis, and nonalcoholic steatohepatitis (reviewed in Levy, 2016). Ocaliva, or obeticholic acid, is a bile acid analogue that was recently approved as a first-in-class FXR agonist (May 2016) for the treatment of primary biliary cholangitis (USFDA, 2016). Ocaliva has an EC_{50} of 99 nM, as determined with ligand-dependent recruitment of SRC1 to FXR by fluorescence resonance energy transfer in a cell-free ligand sensing assay (Pellicciari et al., 2002). Similar to ICP, the only approved therapy for primary biliary cholangitis prior to Ocaliva was Ursodeoxycholic acid, which is efficacious for a subset of patients, and is not always well-tolerated. Ocaliva is also in Phase 2 and 3 trials for the treatment of nonalcoholic steatohepatitis with fibrosis, and primary sclerosing cholangitis (clinicaltrials.gov). Because of both ethical and financial reasons, pursuing a compound for FDA approval in pregnant women alone is not likely to be pursued by pharmaceutical companies. However, the introduction of FXR agonists into the market represents a first step towards understanding their efficacy and safety for potential use for pregnant women with cholestasis.

Overall Conclusions and Implications

The findings described in this dissertation demonstrate a temporal regulation of intestinal nuclear receptor signaling late in gestation in mice that can be mimicked in human intestinal cells treated with steroid hormones. Further, activation of one particular bile acid nuclear receptor, Fxr, ameliorates pro-cholestatic gene adaptations observed in the intestine-liver axis in mice with healthy pregnancies. These data provide a better understanding of drug disposition in pregnancy, as well as the pathogenesis of ICP, and the overall “procholestatic” state of pregnancy that sensitizes women to acquiring liver

disease. These data are necessary for developing more effective treatments to manage maternal disease and prevent fetal complications.

APPENDIX 1: DIFFERENTIAL FMO3 GENE EXPRESSION IN VARIOUS LIVER INJURY MODELS INVOLVING HEPATIC OXIDATIVE STRESS IN MICE

Swetha Rudraiah[a], Jamie E. Moscovitz[b], Ajay C. Donepudi[d], Sarah N. Campion[c], Angela L. Slitt[d], Lauren M. Aleksunes[b], José E. Manautou[a]

[a] Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT, USA

[b] Department of Pharmacology and Toxicology, Rutgers UniversityErnest Mario School of Pharmacy, Piscataway NJ, USA

[c] Drug Safety Research and Development, Pfizer, Inc., Groton, USA

[d] Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI, USA

A-1.1 Abstract

Flavin-containing monooxygenase-3 (FMO3) catalyzes metabolic reactions similar to cytochrome P450 monooxygenase however, most metabolites of FMO3 are considered non-toxic. Recent findings in our laboratory demonstrated *Fmo3* gene induction following toxic acetaminophen (APAP) treatment in mice. The goal of this study was to evaluate *Fmo3* gene expression in diverse other mouse models of hepatic oxidative stress and injury. *Fmo3* gene regulation by Nrf2 was also investigated using Nrf2 knockout (Nrf2 KO) mice. In our studies, male C57BL/6J mice were treated with toxic doses of hepatotoxicants or underwent bile duct ligation (BDL, 10d). Hepatotoxicants included APAP (400 mg/kg, 24 to 48 h), alpha-naphthyl isothiocyanate (ANIT; 50 mg/kg, 2 to 48 h), carbon tetrachloride (CCl_4; 10 or 30 μL/kg, 24 and 48 h) and allyl alcohol (AlOH; 30 or 60 mg/kg, 6 and 24 h). Because oxidative stress activates nuclear factor (erythroid-derived 2)-like 2 (Nrf2), additional studies investigated *Fmo3* gene regulation by Nrf2 using Nrf2 knockout (Nrf2 KO) mice. At appropriate time-points, blood and liver samples were collected for assessment of plasma alanine aminotransferase (ALT) activity, plasma and hepatic bile acid levels, as well as liver Fmo3 mRNA and protein expression. Fmo3 mRNA expression increased significantly by 43-fold at 12 h after ANIT treatment, and this increase translates to a 4-fold change in protein levels. BDL also increased Fmo3 mRNA expression by 1899-fold, but with no change in protein levels. Treatment of mice with CCl_4 decreased liver *Fmo3* gene expression, while no change in expression is detected with AlOH treatment. Nrf2 KO mice are more susceptible to APAP (400 mg/kg, 72 h) treatment compared to their wild-type (WT) counterparts, which is evidenced by greater plasma ALT activity. Fmo3 mRNA and protein expression increased in Nrf2 KO mice after APAP treatment. Collectively, not all hepatotoxicants that produce oxidative stress alter *Fmo3* gene expression. Along with APAP, toxic ANIT treatment in mice markedly increases *Fmo3* gene expression. While BDL increases Fmo3 mRNA

expression, protein level does not change. The discrepancy with *Fmo3* induction in cholestatic models, ANIT and BDL, is not entirely clear. Results from Nrf2 KO mice with APAP suggest that the transcriptional regulation of *Fmo3* during liver injury may not involve Nrf2.

Non-standard Abbreviations:

Flavin-containing monoxygenase-3 (Fmo3); Acetaminophen (APAP); Alpha-naphthylisothiocyanate (ANIT); Bile duct ligation (BDL); Carbon tetrachloride (CCl_4); Allyl alcohol (AlOH); Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)

A-1.2 Introduction

Drug-induced liver injury (DILI) is a major problem for both drug development and clinical care. It accounts for more than 50% of all acute liver failure cases in the U.S. (Larson et al., 2005; Lee et al., 2006). Many chemicals, such as acetaminophen (APAP), carbon tetrachloride (CCl_4) and allyl alcohol (AlOH) have been used to model hepatotoxicity relevant to human exposure. Alpha-naphthyl isothiocyanate (ANIT) and bile duct ligation (BDL) on the other hand are used to model cholestasis, a pathological condition caused by impairment of hepatic bile flow. While ANIT produces intrahepatic cholestasis, BDL produces extrahepatic cholestasis. With APAP and CCl_4, the parent compound is metabolized by cytochrome P450 (CYP) to generate reactive metabolites, N-acetyl-*p*-benzoquinone imine (NAPQI) and tricholoromethyl radical ($^{\cdot}CCl_3$), respectively. AlOH in turn is metabolized in the liver by alcohol dehydrogenase to its reactive metabolite, acrolein. The glutathione adduct of acrolein is converted by CYPs to glycidaldehyde. Toxicity resulting from these reactive metabolites is multifactorial and includes lipid peroxidation, generation of oxidative stress, altered cellular redox status and protein adduct formation (Ohno et al., 1985; Cohen et al., 1997; Burcham and Fontaine, 2001; Jaeschke et al., 2012). During cholestasis resulting from either BDL or ANIT treatment, increase in bile acid concentration stimulates production of reactive oxygen species eventually leading to hepatocellular necrosis and apoptosis (Trauner et al., 1998b; Sokol et al., 2001).

The role of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) role as a master defense against hepatotoxicity produced by various chemicals has been investigated in several studies. Nrf2 belongs to the cap ‘n’ collar family of transcription factors that promotes transcription of a battery of cytoprotective genes (Aleksunes and Manautou, 2007; Kensler et al., 2007). Under basal conditions, Nrf2 is largely bound to the cytoskeletal

anchoring protein Kelch-like ECH-associated protein 1 (Keap1) also known as cytosolic Nrf2 inhibitor in the cytoplasm. In response to oxidative stress, Nrf2 is released from Keap1 that translocates into the nucleus. In the nucleus, Nrf2 binds to the GTGACA***GC core sequence of the antioxidant response element (ARE) (Rushmore et al., 1991) and promotes ARE-mediated antioxidant gene expression.

A low toxic APAP dose causes nuclear accumulation of Nrf2 in mouse liver, which is accompanied by increased expression of Nrf2 dependent cytoprotective genes such as heme oxygenase-1 (*Hmox1*), NAD(P)H:quinone oxidoreductase-1 (*Nqo1*) and glutamate cysteine ligase catalytic subunit (*Gclc*) (Bauer et al., 2000; Chiu et al., 2002; Goldring et al., 2004; Aleksunes et al., 2005; Aleksunes et al., 2006b). Similar results have been reported with ANIT, BDL, CCl_4 and AlOH, other models of hepatic oxidative stress used in the present study (Aleksunes et al., 2005; Aleksunes et al., 2006b; Randle et al., 2008; Tanaka et al., 2009; Liu et al., 2013). On the other hand, Nrf2 KO mice are more susceptible to APAP-induced liver injury compared to their wild-type counterparts (Chan et al., 2001; Enomoto et al., 2001). Likewise, Nrf2 KO mice are also more susceptible to CCl_4- and AlOH-induced hepatoxicity compared to wild-type mice (Liu et al., 2013). However, Nrf2-null mice do not exhibit any difference in susceptibility to either BDL or ANIT treatment. This response is attributed to the adaptive compensatory changes involving nuclear transcription factors, including Fxr, Shp, Pxr and Hnf1α, efflux bile acid transporters, altered GSH levels and bile flow rates in Nrf2 KO mice (Tanaka et al., 2009; Weerachayaphorn et al., 2012). Collectively, the models of hepatic injury selected for the current study not only result in hepatic oxidative stress but also activate the Nrf2-Keap1 regulatory pathway.

Despite *Fmo3* being considered non-inducible, studies with aryl hydrocarbon receptor (AhR) agonists in mice show liver *Fmo3* gene induction (Celius et al., 2008; Celius et al., 2010). A recent gene array analysis performed in our laboratory also demonstrated *Fmo3* gene induction in the APAP autoprotection mouse model (mice receiving a low hepatotoxic APAP dose are resistant to a subsequent higher APAP dose) (O'Connor et al., 2014). Unlike with AhR agonists that result in marginal increases in Fmo3 protein expression in mouse liver, we show significant increases in Fmo3 protein levels in APAP autoprotected mice (Rudraiah et al., 2014). *Fmo3* induction by other hepatotoxicants that produce oxidative stress is not currently known.

In human liver, transcription factors regulating constitutive *FMO3* expression as well as those involved in developmental expression pattern are extensively studied (Klick and Hines, 2007; Klick et al., 2008; Shimizu et al., 2008). Because the mammalian FMOs were considered non-inducible by xenobiotics (Cashman and Zhang, 2002; Krueger and Williams, 2005), the transcriptional regulation of FMO involving stress-activated transcription factors or receptors that bind ligands and interact with DNA was not studied as other forms of regulation. Thus, little is known about the transcriptional regulation of Fmo3 in response to toxicant exposure. Recently, Celius et al. (2010) show that the Fmo3 mRNA up-regulation by 3-methylcholanthrene (3MC) and benzo(a)pyrene (BaP) but not TCDD in Hepa-1 cells is mediated by p53 and its binding to a p53-response element in the promoter region of Fmo3 (Celius et al., 2010).

Differentially expressed genes in the APAP autoprotection model were further analyzed using Causal Reasoning Engine (CRE), a recently developed computation platform (O'Connor et al., 2014). CRE analysis provides hypotheses on the upstream molecular events that best explain gene expression profiles based on prior biological knowledge.

CRE analysis of differentially expressed genes in APAP autoprotection study supports an induction of the Nrf2 pathway (O'Connor et al., 2014). Additionally, the 5'-flanking region of the mouse *Fmo3* contains multiple copies of the ARE (Celius et al., 2008). Therefore, the purpose of the present study was to investigate liver *Fmo3* gene expression under oxidative stress conditions involving activation of the Nrf2-Keap1 regulatory pathway. Mice were dosed with hepatotoxicants APAP (400 mg/kg, 24 to 48 h), ANIT (50 mg/kg, 2 to 48 h), CCl_4 (10 or 30 μL/kg, 24 and 48 h) or AlOH (30 or 60 mg/kg, 6 and 24 h) or underwent sham surgery or bile duct ligation (10 d). Doses selected for hepatotoxicants are based upon previous studies conducted in our laboratory resulting in oxidative stress and tissue injury. The inclusion of multiple time-points following hepatotoxicants exposure enabled comprehensive characterization of temporal changes in *Fmo3* in relation to injury and recovery. Further, in order to investigate whether Nrf2 mediates *Fmo3* gene expression, Nrf2 KO mice were employed. APAP was used as a model toxicant in the Nrf2 KO mice study. From these experiments, it is concluded that not all hepatotoxicants that produce oxidative stress in mice induce liver *Fmo3* gene expression. Toxic ANIT treatment, along with the previously demonstrated APAP treatment, markedly increases *Fmo3* gene expression. While BDL increases Fmo3 mRNA expression, protein levels do not change. APAP treatment induces *Fmo3* gene expression in Nrf2 KO mice liver suggesting that the transcriptional regulation of *Fmo3* might not involve Nrf2.

A-1.3 Materials and methods

Chemicals

Acetaminophen, alpha-naphthyl isothiocyanate, carbon tetrachloride, allyl alcohol, propylene glycol and corn oil were purchased from Sigma-Aldrich (St Louis, MO). All other reagents were of reagent grade or better and commercially available.

Animals

Male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Nine- to ten-week old mice were used for this study. Upon arrival, mice were acclimated for one week prior to experimentation. Mice were housed in a temperature-, light- and humidity-controlled environment. Mice were fed laboratory rodent diet (Harlan Teklad 2018, Madison, WI) *ad libitum*.

Experimental design 1. Following an overnight fast, male mice (n=6) were treated with APAP (400 mg/kg in 50% propylene glycol, ip, 24, 48 and 72 h), ANIT (50 mg/kg in corn oil, po, 2, 4, 8, 12, 24 and 48 h), CCl_4 (10 or 30 μL/kg in corn oil, ip, 24 and 48 h), AlOH (30 or 60 mg/kg in saline, ip, 6 and 24 h) or vehicle. At the end of each experiment, animals were sacrificed at the specified time-points for each hepatotoxicant and plasma and livers were collected for analysis. BDL liver samples were obtained from our collaborator Dr. Slitt's laboratory. Briefly, sham or BDL surgery was performed under phenobarbital-induced anesthesia (65 mg/kg, ip). The surgeries were performed at the University of Rhode Island, College of Pharmacy animal facility with IACUC approval. Blood and liver were collected 10 d after surgery for analysis.

Experimental design 2. Following overnight fasting, male Nrf2KO mice and their wild-type counterparts (C57BL/6J) were treated with APAP (400 mg/kg, ip) in 50% PG or vehicle. Plasma and livers were collected 72 h after APAP treatment for analysis.

All animal studies were performed in accordance with National Institute of Health standards and the Guide for the Care and Use of Laboratory Animals. This work was approved by the University of Connecticut's Institutional Animal Care and Use Committee.

Alanine aminotransferase (ALT) assay

Plasma or serum ALT activity was determined as a biochemical indicator of hepatocellular injury. Infinity ALT Liquid Stable Reagent (Thermo Fisher Scientific Inc., Waltham, MA) was used to determine ALT activity. Samples were analyzed using a Bio-Tek Power Wave X Spectrophotometer.

Total bile acid assay

Total bile acids were extracted from whole liver homogenates using a t-butanol extraction method. Briefly, livers were homogenized in extraction solution (1:1, water: t-butanol) and bile acids extracted overnight at room temperature in the dark. Plasma or serum and hepatic bile acid levels were measured using a spectrophotometric bile acid assay kit (Bioquant, San Diego, CA) according to manufacturer's protocol.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from mouse liver samples using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was then reverse-transcribed into cDNA using an M-MLV RT kit (Invitrogen, Carlsbad,

CA). Fmo3 mRNA expression was quantified by the ΔΔCT method and normalized to two housekeeping genes, β-actin and ribosomal protein S18. Data presented were normalized to β-actin. Primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA) and are as follow: Fmo3 forward: 5′-GGA AGA GTT GGT GAA GAC CG-3′, reverse: 5′-CCC ACA TGC TTT GAG AGG AG-3′. Amplification was performed using an Applied Biosystems 7500 Fast Real-Time PCR System. Amplification was carried out in a 20 μL reaction volume containing 8 μL diluted cDNA, Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 1 μM of each primer.

Preparation of microsomal fraction and western blot analysis

Microsomes were isolated from livers as described previously (Cashman and Hanzlik, 1981; Rudraiah et al., 2014) and stored at -80°C until use. Protein concentration was determined by the method of Lowry using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA). For western blot analysis, microsomal proteins (10 μg) were electrophoretically resolved using 10% polyacrylamide gels and transferred onto PVDF-Plus membrane (Micron Separations, Westboro, MA). A rabbit anti-mouse Fmo3 primary antibody (GenScript USA Inc., NJ) (1:5000) was used to detect Fmo3 with β-actin as a loading control. Blots were then incubated with HRP conjugated secondary antibodies against rabbit IgG (1:2000). Protein-antibody complexes were detected using a chemiluminescent kit (Thermo Scientific, IL) followed by exposure to X-ray film.

Enzyme assay

Methimazole (MMI) metabolism was determined spectrophotometrically by measuring the rate of MMI *S*-oxygenation via the reaction of the oxidized product with nitro-5-thiobenzoate (TNB) to generate 5,5'-dithiobis(2-nitrobenzoate) (DTNB). The incubation mixture consisted of 50 mM sodium phosphate buffer (pH 9.0), 0.5 mM NADP+, 0.5 mM

glucose-6-phosphate, 1.5 IU/mL glucose-6-phosphate dehydrogenase, 0.06 mM DTNB, 0.04 mM dithiothreitol and 100 to 150 µg/mL liver microsomes isolated from mice. Reactions were initiated by the addition of different amounts of MMI (substrate), with a concentration range from 1.25 to 800 µM. Incubations were done in duplicates. The disappearance of the yellow color was measured spectrophotometrically at 412 nM and specific activity (µM/min/mg) was determined using the molar extinction coefficient of NADPH (28.2 mM-1cm-1).

Statistical analysis

The statistical significance between groups was determined using the Student's t-test, one-way ANOVA with Dunnett's post-hoc test or two-way ANOVA followed by the Bonferroni's post-hoc test. While Student's t-test was used to compare means of two different treatment groups, ANOVA was used to compare the means of more than two treatment groups that are normally distributed with a common variance. All statistical analysis was performed using GraphPad Prism version 4.00 for Macintosh (GraphPad Software, Inc., San Diego, CA). Data are presented as mean ± standard error (SE), with $p<0.05$ considered statistically significant.

A-1.4 Results

Plasma ALT activity in the mouse liver injury models.

The hepatotoxicity of APAP (400 mg/kg) at 24, 48 and 72 h as assessed by plasma ALT activity has been reported previously (Rudraiah et al., 2014). Briefly, APAP increased plasma ALT activity to 191±18 and 219±47 IU/L at 24 and 48 h, respectively (mean plasma ALT activity in control mice was 25±5 IU/L). Plasma ALT activity is not statistically different from vehicle controls by 72 h, indicating recovery from APAP-induced liver injury. Plasma ALT activity in all mouse models of liver injury is shown in Fig. A-1.1. ANIT increased ALT activity at 12 h (182±9 IU/L), which continued to increase at 24 and 48 h (715±126 IU/L and 781±45 IU/L, respectively) compared to vehicle control group (9±1 IU/L). BDL for 10 d results in an elevation of ALT activity to 185±10 IU/L. A high CCl_4 dose (30 µL/kg) increased plasma ALT at 24 and 48 h to 6367±1135 and 397±111 IU/L, respectively. A low CCl_4 dose (10 µL/kg) also increased plasma ALT activity at 24 and 48 h to 757±106 and 416±68 IU/L, respectively. The greatest increase in plasma ALT with both doses of CCl_4 is observed at 24 h. While AlOH treatment (low dose, 30 mg/kg) did not result in significant increases in ALT levels at 6 h and 24 h (36±4 and 73±24 IU/L, respectively), a high AlOH dose (60 mg/kg) results in significantly higher ALT levels at both 6 h and 24 h (153±50 and 4440±2428 IU/L, respectively). The associated histopathological damage with all hepatotoxicants has been reported previously (Aleksunes et al., 2005; Aleksunes et al., 2006a; Campion et al., 2009). With acute toxicity models, plasma ALT activity lesser than 1000 IU/L is usually associated with a minimal to mild hepatocellular damage (histological grade of 2 or less than 2), and an ALT activity higher than 1000 IU/L is associated with moderate, marked or severe hepatocellular damage (histological grade of 3, 4, or 5) (Manautou et al., 1994; Aleksunes et al., 2008a). Alternatively, a 10 d BDL with an associated ALT

activity of less than 200 IU/L, exhibit a chronic hepatocellular damage involving fibrosis (Donepudi et al., 2012).

Hepatic Fmo3 mRNA expression in the mouse liver injury models.

Fmo3 mRNA levels were quantified by qRT-PCR and the results are presented in Fig. A-1.2. Fmo3 mRNA expression following 400 mg/kg APAP treatment has been previously reported (Rudraiah et al., 2014). Briefly, Fmo3 mRNA levels increased by 5±2.6- and 23±5.6-fold, at 24 and 48 h after APAP, respectively, compared to the 0 h control group. ANIT increased Fmo3 mRNA levels as early as 2 h after treatment. This increase peaked and is statistically significant only at 12 h (43±10-fold increase), compared to 0 h vehicle control group. BDL also increased Fmo3 mRNA expression by 1899±625-fold, compared to sham operated mice. Both the low and high dose of CCl_4 decreased Fmo3 mRNA levels at both time points examined, and this decrease is statistically significant only at 48 h (10 µL/kg: 0.2±0.06-fold and 30 µL/kg: 0.2±0.08-fold). No change in liver Fmo3 mRNA levels is observed with AlOH treatment.

Hepatic bile Acid concentrations in the mouse liver injury models.

To determine the extent of cholestasis induced by different hepatotoxicants used in the current study, total hepatic bile acid levels were measured (Fig. A-1.3). As expected ANIT increased hepatic bile acid levels to 1.7±0.2 and 1.6±0.4 µmol/g at 24 and 48 h, respectively, compared to 0 h vehicle control group (0.2±0.02 µmol/g). BDL also increased hepatic bile acid concentrations to 1949±85 µmol/100 mg compared to sham operated group (1061±165 µmol/100 mg). No significant changes were observed in mice treated with APAP, CCl_4 and AlOH.

Bile acid concentrations in the mouse liver injury models.

Plasma bile acid concentration is also a biomarker of cholestasis. In order to determine the extent of cholestasis, plasma bile acid levels were also quantified in all mouse models of liver injury (Fig. A-1.4). Plasma bile acid concentration increased in ANIT treated mice at 24 and 48 h to 210±20 and 330±128 µmol/L, respectively, compared with vehicle treated mice (2±0.2 µmol/L). Bile duct ligation significantly increased serum bile acid levels to 1359±142 µmol/L compared to sham operated mice (86±11 µmol/L). Significant increases in plasma bile acid concentrations are seen in high dose CCl_4 treated mice at both 24 and 48 h (112±15 µmol/L and 29±2 µmol/L, respectively). Exposure to APAP and AlOH did not result in any significant change in plasma bile acid levels.

Fmo3 protein expression in the mouse liver injury models.

Temporal expression of the Fmo3 protein following exposure to hepatotoxicants and BDL were quantified by western blotting. Representative blots and associated densitometric analyses are shown in Fig. A-1.5. Fmo3 protein increase following APAP treatment has been previously reported (Rudraiah et al., 2014). Although the expression of Fmo3 protein tended to increase by 1.1±0.3- to 1.6±0.2-fold between 24 and 72 h after APAP, the increase is statistically significant only at 72h. Consistent with Fmo3 mRNA changes evidenced with ANIT treatment, Fmo3 protein levels also tend to increase as early as 2 h. However, this increase is significant only at 12 h (3.5±0.9-fold) compared to 0 h vehicle treated group. Fmo3 protein levels decreased to 0.7±0.08- and 0.3±0.1-fold at 24 and 48 h, respectively, after exposure to ANIT. Conversely, in spite of the dramatic increase in Fmo3 mRNA levels after BDL, Fmo3 protein levels did not change. No significant changes in Fmo3 protein expression are observed with CCl_4 or AlOH treatment.

Plasma ALT activity and Fmo3 mRNA levels after APAP treatment in wild-type and Nrf2 knockout mice.

To investigate whether Nrf2 mediates *Fmo3* gene expression, Nrf2 KO and WT mice were administered APAP (400 mg/kg) in 50% propylene glycol. APAP was used as a model toxicant for Nrf2 activation. Administration of 400 mg/kg APAP to male C57Bl/6J WT mice did not result in significant elevation of plasma ALT levels at 72 h. This is consistent with the previously reported results, indicating recovery from APAP-induced liver injury and increased Fmo3 protein expression in WT mice (Rudraiah et al., 2014). In contrast, plasma ALT activity is elevated at 72 h in Nrf2 KO mice receiving the same dose of APAP (176±12 IU/L) compared to vehicle control group (23±5 IU/L) (Fig. A-1.6A). This is again consistent with the literature and confirms that Nrf2 plays an important role not only in the magnitude of toxicity, but also in recovery from APAP-induced liver injury. Fmo3 mRNA levels were quantified by qRT-PCR. The results in Fig. A-1.6B show that there is no change in Fmo3 mRNA levels at 72 h in wild-type mice administered 400 mg/kg APAP. This is again consistent with previous reports, where maximal Fmo3 mRNA expression is seen at 48 h after APAP administration and returns to normal by 72 h (Rudraiah et al., 2014). Notably, Fmo3 mRNA expression is significantly higher in Nrf2 KO mice 72 h after APAP treatment by 140±43-fold change compared to vehicle control group.

Fmo3 protein expression after APAP treatment in wild-type and Nrf2 knockout mice.

To examine the temporal changes in *Fmo3* gene expression following APAP treatment in WT and Nrf2 KO mice, Fmo3 protein levels were quantified by western blotting and by measuring catalytic activity using MMI as substrate. Representative blots and associated densitometric analysis are shown in Fig. A-1.7A. Consistent with increased Fmo3 mRNA

expression in Nrf2 KO mice administered APAP (400 mg/kg) at 72 h, Fmo3 protein levels are also significantly higher (5.1±1.3-fold) compared to vehicle control group. Measuring FMO catalytic activity using MMI can also quantitate Fmo3 protein induction (Zhang et al., 2007). FMO specific activity increased significantly in Nrf2 KO livers (48±6 μM/min/mg) at 72 h after APAP (Fig. A-1.7B).

A-1.5 Discussion

FMO3 is a microsomal monooxygenase enzyme involved in the oxygenation of lipophilic substrates to more polar metabolites. Substrates include nitrogen-, sulfur- and phosphorous-containing drugs and xenobiotics, and most metabolic products of Fmo3 are considered to be non-toxic (Krueger and Williams, 2005). Although FMOs were discovered in the 1960s (Miller et al., 1960) and further purified in 1972 (Ziegler and Mitchell, 1972), it was not until 2008 that enzyme induction by xenobiotics was demonstrated (Celius et al., 2008; Celius et al., 2010). In these studies, in spite of a very large increase in Fmo3 mRNA level by AhR agonist treatment, only a modest increase in protein level and function was reported. A gene array analysis performed in our laboratory also demonstrated *Fmo3* gene induction in the mouse model of APAP autoprotection (O'Connor et al., 2014). In this APAP autoprotection mouse model, we show a significant increase in Fmo3 protein expression and function (Rudraiah et al., 2014). Constitutive Fmo3 expression in a female mouse liver is localized in the areas surrounding the periportal region (Janmohamed et al., 2004; Rudraiah et al., 2014). Following APAP exposure, the Fmo3 protein expression in APAP autoprotected livers is observed in the centrilobular regions where APAP-induced damage and/or hepatocellular compensatory proliferation is seen. Furthermore, we show that the enhanced expression of Fmo3 confers resistance against APAP-induced hepatotoxicity in mice (Rudraiah et al., 2014).

In the present study, the effect of various other hepatotoxicants on *Fmo3* gene expression in male C57BL/6J mouse liver was examined. A unifying theme for all hepatotoxicants used in our study is the oxidative stress. These models of oxidative stress used are very well studied with respect to activation of Nrf2-Keap1 regulatory pathway. This has been repeatedly demonstrated in our laboratory and in the literature

(Bauer et al., 2000; Chiu et al., 2002; Goldring et al., 2004; Aleksunes et al., 2005; Aleksunes et al., 2006a; Aleksunes et al., 2006b; Randle et al., 2008; Tanaka et al., 2009; Liu et al., 2013). We were confident that the treatments selected result in oxidative stress. Therefore, markers of oxidative stress with each experiment are not determined.

Coincidentally all of these produce cholestasis, with an exception of AlOH (Tanaka et al., 2009; Aleksunes et al., 2012; Donepudi et al., 2012; Weerachayaphorn et al., 2012; Yamazaki et al., 2013). Furthermore, perturbation of bile acid homeostasis has been demonstrated to be an early event in the pathogenesis of drug induced liver injury (Yamazaki et al., 2013). To determine whether accumulation of bile acids is a signaling event regulating Fmo3, we measured total hepatic and plasma bile acid levels. Consistent with the literature, ANIT and BDL increased plasma and hepatic bile acid concentrations. A high CCl_4 dose did not significantly alter liver bile acid levels, but significantly increased plasma bile acid concentration. APAP tended to increase both hepatic and plasma bile acid concentrations, but this is not statistically significant.

In general, ANIT and BDL-mediated damage significantly increased liver Fmo3 mRNA expression. While CCl_4-mediated liver injury significantly decreased Fmo3 mRNA expression, no change in expression is evidenced with AlOH. Fmo3 mRNA expression in response to ANIT treatment precedes serum and hepatic bile acid accumulation and the maximal *Fmo3* expression (both mRNA and protein) parallels mild hepatocellular damage. With BDL, unlike ANIT, *Fmo3* mRNA induction is associated with higher serum and hepatic bile acid levels, but protein level does not change.

Overt ANIT-induced hepatocellular damage at 24 and 48 h decreased *Fmo3* gene expression and the decease is statistically significant at 48 h compared to 12 h ANIT-

treated group. This observation with respect to the relationship between plasma ALT level and *Fmo3* gene expression is consistent with our results with APAP hepatotoxicity. We have demonstrated in our APAP autoprotection mouse model that the APAP (400 mg/kg) pretreated group as well as the autoprotected group (APAP pretreatment: 400 mg/kg, APAP challenge: 600 mg/kg) exhibit average plasma ALT values of about 250 IU/L and *Fmo3* protein induction. The group that receives a toxic APAP dose of 600 mg/kg exhibits much greater average plasma ALT activity of about 1600 IU/L, but no *Fmo3* gene induction (Rudraiah et al., 2014).

One key feature that is consistent with APAP-, BDL- and ANIT-induced liver injury and *Fmo3* gene expression is plasma ALT elevations. Mild ALT elevation is the common feature for all three models where Fmo3 mRNA induction is observed. Although BDL is a chronic injury model, it well known that the ALT values do not correlate well with the severity of liver damage particularly during fibrotic hepatocellular necrosis (Kallai et al., 1964). A similar clinical feature is also seen in cases of primary biliary cirrhosis (BDL models primary biliary cirrhosis in humans) (Hohenester et al., 2009). The lack of Fmo3 protein detection during BDL may be due to signal dilution by fibrotic liver tissue. It is also possible that under oxidative stress conditions some proteins involved in translation are oxidized *in vivo* inhibiting translation (Shenton et al., 2006). To add to this complexity, in spite of lower plasma ALT activity at 24 h (low dose CCl_4), and 48 h (both high and low dose CCl_4) with CCl_4, there is down-regulation of *Fmo3* gene expression. In AlOH-treated livers, lower plasma ALT activity did not show any change in *Fmo3* gene expression. This is suggestive of an unknown underlying mechanism unique to APAP, ANIT and BDL, which is contributing to *Fmo3* mRNA induction. Studies are necessary to investigate the regional distribution of Fmo3 protein expression during ANIT- and BDL-induced liver injury to determine whether the protein expression in these models is

localized to the portal vein, where ANIT- and BDL-induced hepatotoxicity is confined. Collectively, these data suggest that there is a fine-tuning with regard to the bile acid level, hepatocellular damage, and *Fmo3* gene expression.

As discussed before, all models of hepatic injury used in the current study result in hepatic oxidative stress and activate the Nrf2-Keap1 regulatory pathway. Importantly, CRE analysis of differentially expressed genes in our APAP autoprotection study supported an induction of the Nrf2 pathway (O'Connor et al., 2014). Additionally, Celius *et al.* (2008) showed that the 5'-flanking region of the mouse *Fmo3* contains multiple copies of the ARE (Celius et al., 2008). Promoter analysis of mouse *Fmo3* promoter (7 kb length) using MatInspector software from Genomatix BiblioSphere, also showed an ARE at about 3 kb from the transcription start site (data not shown). We also found two other binding sites for Bach1, at 600 bp and 2 kb from the *Fmo3* transcription start site (data not shown). Bach1 is a regulatory mediator of Nrf2, in that it is a transcription repressor. Bach1 heterodimerizes with small Maf proteins in the absence of cellular stress and represses gene expression. In the presence of oxidative stress, Bach1 is released from the Maf proteins and is replaced by Nrf2 (Kaspar et al., 2009). Thus, using APAP as a model toxicant for Nrf2 activation, *Fmo3* gene expression was evaluated in Nrf2 KO mice. Compared to APAP-treated wild-type mice, Nrf2 KO mice exhibit persistent and significant hepatocellular damage 72 h after APAP administration. Nrf2 KO mice are not only more susceptible to APAP-induced hepatocellular necrosis, but also fail to recover from injury as rapidly as the WT mice. This is consistent with the literature that describes Nrf2 as a master regulator of many cytoprotective genes involved in APAP-induced hepatotoxicity (Bauer et al., 2000; Chiu et al., 2002; Goldring et al., 2004; Aleksunes et al., 2005; Aleksunes et al., 2006a). Finally, *Fmo3* gene induction by APAP treatment in Nrf2 null mice suggests that the transcriptional

regulation of Fmo3 does not involve this transcription factor. The persistent mild hepatocellular injury and oxidative stress in Nrf2 KO mice most likely activates other signaling mechanisms involved in *Fmo3* gene induction.

Future studies will investigate the role of other nuclear receptors in *Fmo3* gene induction. Particularly, farnesoid X receptor (FXR) role in *Fmo3* gene induction during APAP hepatotoxicity is worth investigating. FXR is one of the major bile acid sensors in the liver (Chiang, 2002) and plays a protective role during cholestasis development. Recently, it is shown that activation of FXR induces Fmo3 protein function (Bennett et al., 2013). Activation of FXR also provides protection against APAP-induced hepatotoxicity (Lee, 2010). In addition, we discovered three binding sites for farnesoid X receptor-response element (FXRE) at about 1.6, 2.1 and 3.3 kb from the transcription start site on the mouse *Fmo3* promoter. Although, Pregnane X receptor (PXR) is also a bile acid sensor, no binding sites for PXR were found on promoter analysis and furthermore, PXR activation sensitizes APAP-induced hepatotoxicity (Guo et al., 2004; Cheng et al., 2009).

In conclusion, this study comprehensively characterizes for the first time temporal changes in *Fmo3* gene expression during different conditions of hepatic oxidative stress. In particular, we show that toxic ANIT and BDL significantly alter *Fmo3* mRNA expression, but only Fmo3 protein with ANIT. The reason for this discrepancy is not yet clear or easy to rationalize. It is possible that *Fmo3* is also protective during other chemical-induced liver injury including ANIT. Even though the exact mechanism of *Fmo3* gene expression or its role in protecting against toxicant-induced liver injury is not yet clear, the observed toxicity threshold for *Fmo3* gene expression is intriguing. This work

advances the lack of knowledge with regard to the inducibility of *Fmo3* and its potential protective role in drug-induced liver injury.

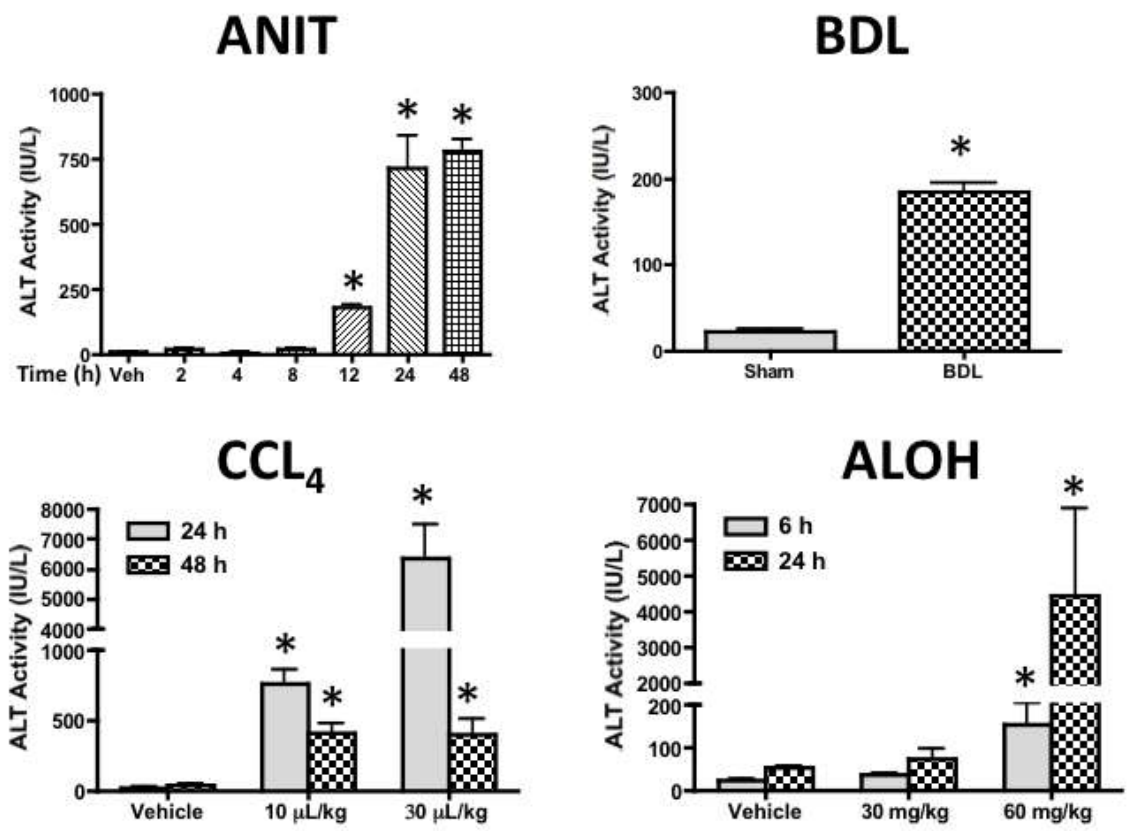


Fig. A-1.1. Plasma ALT activity in mice treated with hepatotoxicants or BDL. Male C57BL/6J mice were treated with ANIT (50 mg/kg, po, 2,4,8,12,24 & 48 h), CCl_4 (10 & 30 µL/kg, ip, 24 & 48 h), AlOH (30 & 60 mg/kg, ip, 6 & 24 h) or BDL (10 d). Plasma was collected from mice at various time points following hepatotoxicant treatment or the appropriate vehicle. The data are presented as mean plasma ALT (IU/L) ± SE. One-way ANOVA, t-test or two-way ANOVA was performed, appropriately, followed by the Dunnett's posttest for One-way ANOVA and the Bonferroni posttest for two-way ANOVA. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle-treated and hepatotoxicant-treated group.

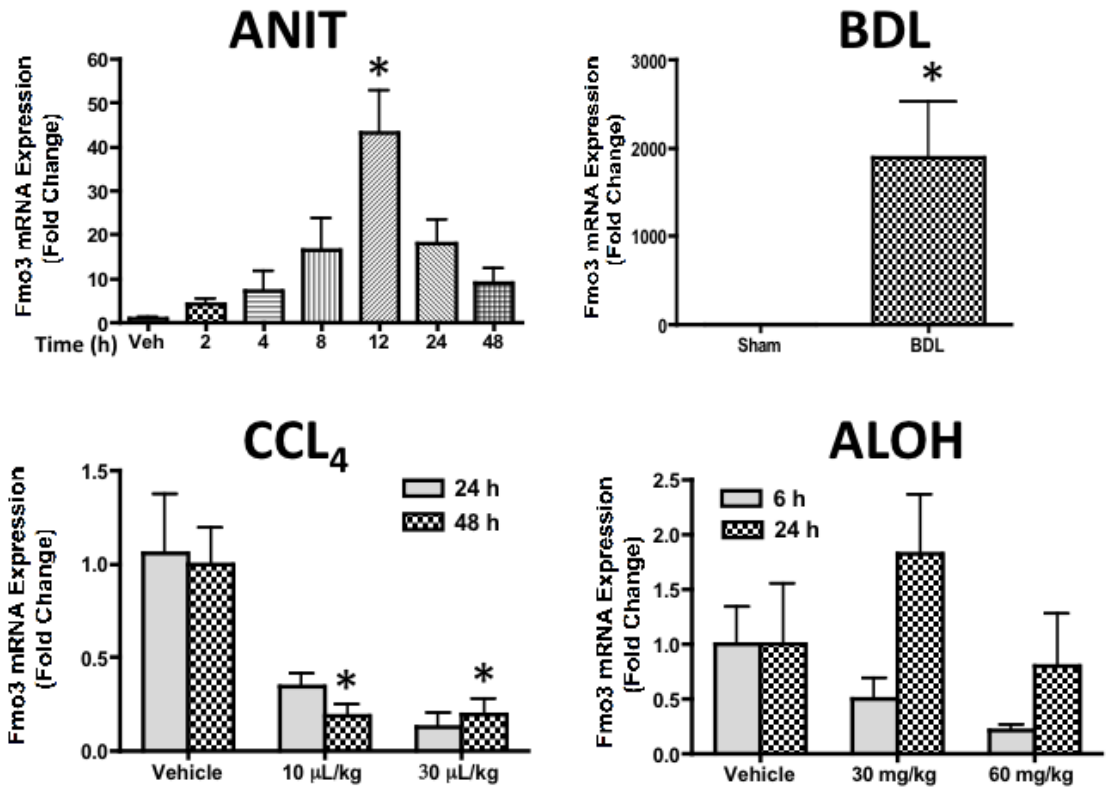


Fig. A-1.2. Quantitative RT-PCR analysis of liver Fmo3 transcripts after hepatotoxicant treatment or BDL. Livers were collected from mice sacrificed at respective time-points (2,4,8,12,24 & 48 h for ANIT, 24 & 48 h for CCl_4, 6 & 24 h for AlOH and 10 d for BDL). RNA was isolated and cDNA was made using a commercial MMLV-RT kit. The cDNA samples were analyzed by quantitative RT-PCR using Fmo3 mouse-specific primers. Gene expression was normalized to the housekeeping gene β-actin. Fmo3 mRNA expression is presented as mean Fold Change ± SE. One-way ANOVA, t-test or two-way ANOVA was performed, appropriately, followed by the Dunnett's posttest for One-way ANOVA and the Bonferroni posttest for two-way ANOVA. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle-treated and hepatotoxicant-treated or BDL group.

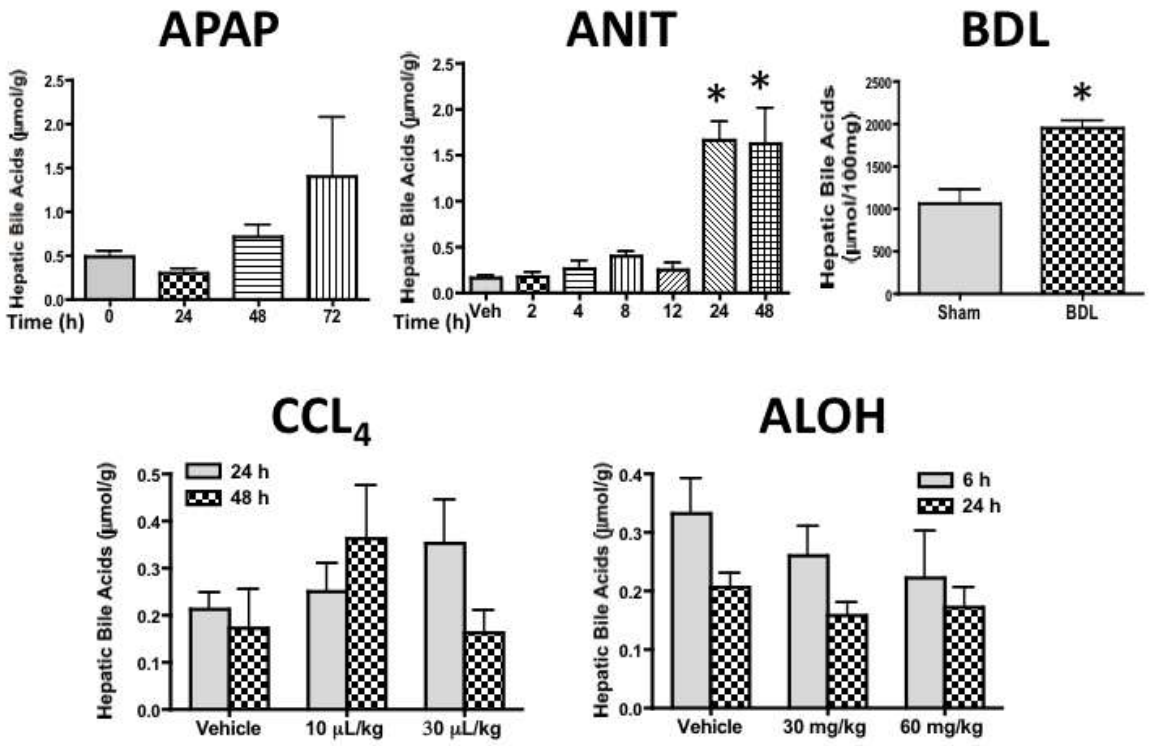


Fig. A-1.3. Hepatic bile acid levels after hepatotoxicant administration or BDL. Livers were collected at the end of each study (24, 48 & 72 h for APAP, 2,4,8,12,24 & 48 h for ANIT, 10 d for BDL, 24 & 48 h for CCl_4 and 6 & 24 h for AlOH). Liver total bile acids were measured spectrophotometrically using a commercial bile acid assay kit. Hepatic total bile acids are expressed as mean hepatic bile acids (µmol/g) ± SE. One-way ANOVA, t-test or two-way ANOVA was performed, appropriately, followed by the Dunnett's posttest for one-way ANOVA and the Bonferroni posttest for two-way ANOVA. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle-treated and hepatotoxicant-treated or BDL group.

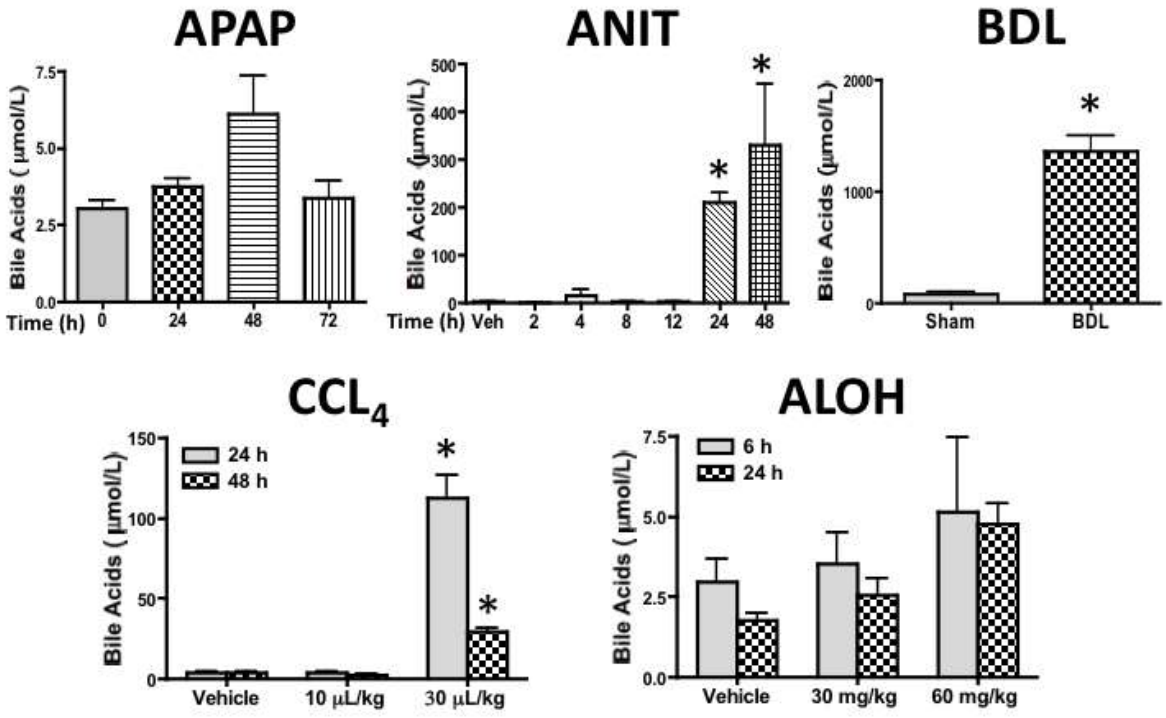


Fig. A-1.4. Bile acid levels in the mouse models of liver injury. Mice were administered APAP (400 mg/kg, ip, 24, 48 & 72 h), ANIT (50 mg/kg, po, 2,4,8,12,24 & 48 h), CCl_4 (10 & 30 µL/kg, ip, 24 & 48 h), AlOH (30 & 60 mg/kg, ip, 6 & 24 h) or BDL (10 d). Blood was collected and the plasma/serum was isolated by centrifugation. Plasma/serum total bile acids were measured spectrophotometrically using a commercial bile acid assay kit. Plasma total bile acids are expressed as mean hepatic bile acids (µmol/L) ± SE. One-way ANOVA, t-test or two-way ANOVA was performed, appropriately,followed by the Dunnett's posttest for one-way ANOVA and the Bonferroni posttest for two-way ANOVA. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle-treated and hepatotoxicant-treated or BDL group.

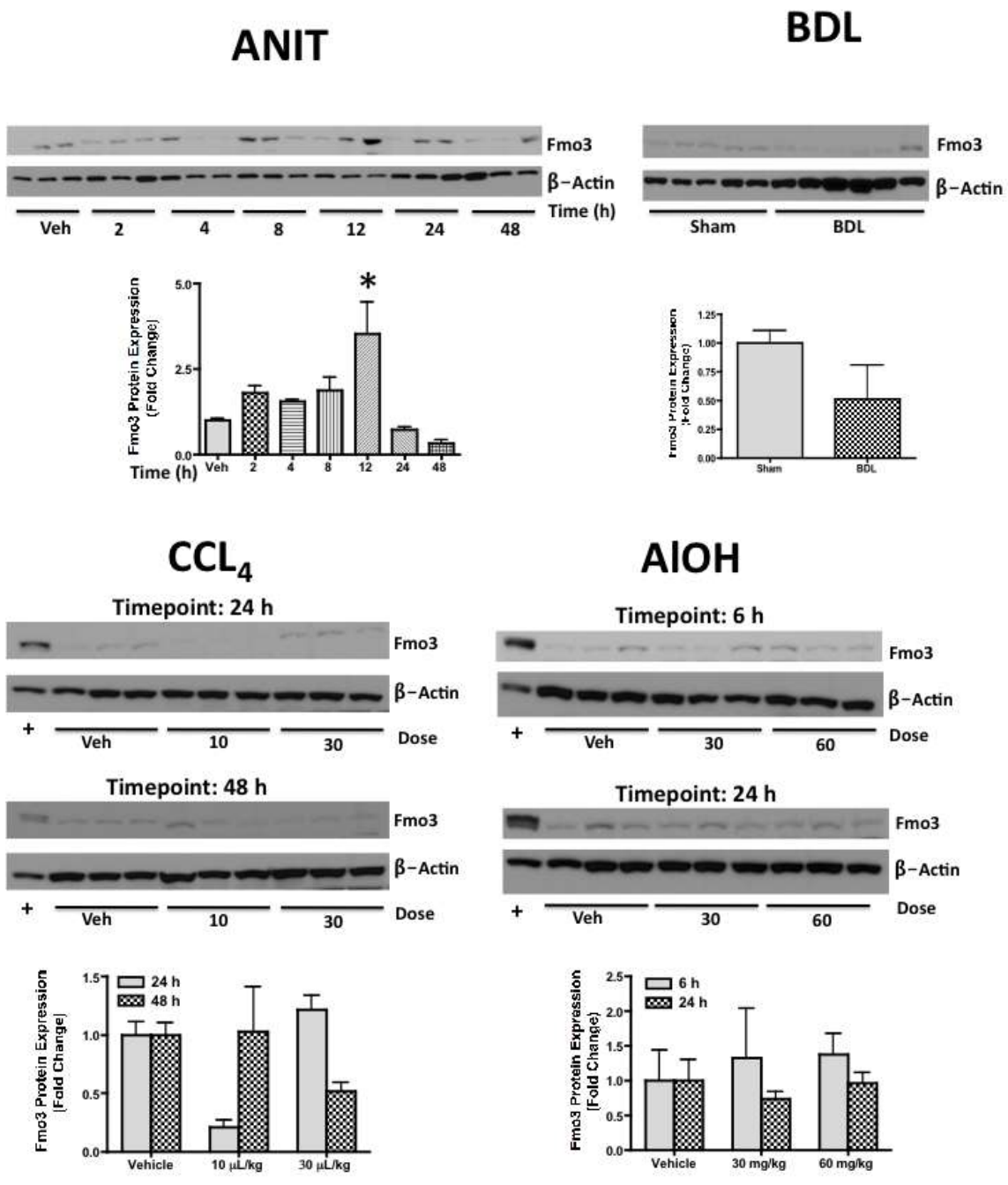

ANIT
BDL
Fmo3
β−Actin
Time (h)
Veh
2
4
8
12
24
48
Sham
BDL
Fmo3 Protein Expression (Fold Change)
*
CCL4
AlOH
Timepoint: 24 h
Timepoint: 48 h
Timepoint: 6 h
Timepoint: 24 h
+
Dose
10
30
60
24 h
48 h
6 h
Vehicle
10 µL/kg
30 µL/kg
30 mg/kg
60 mg/kg

Fig. A-1.5. Analysis of liver Fmo3 protein expression in the mouse models of liver injury by western blotting. Western immunoblots for Fmo3 were performed using liver microsomes from control and hepatotoxicant-treated or BDL mice. A custom-made rabbit anti-mouse Fmo3 primary antibody, described in *Materials and Methods* was used to detect Fmo3. Fmo3 protein levels were normalized to β-actin loading control. Microsomal proteins isolated from naïve female mouse liver was used as a positive control (indicated by "+" sign). The data are presented as blots and as mean Fmo3 protein expression (Fold Change) ± SE. One-way ANOVA, t-test or two-way ANOVA was performed, appropriately, followed by the Dunnett's posttest for One-way ANOVA and the Bonferroni posttest for two-way ANOVA. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle-treated and hepatotoxicant-treated or BDL group.

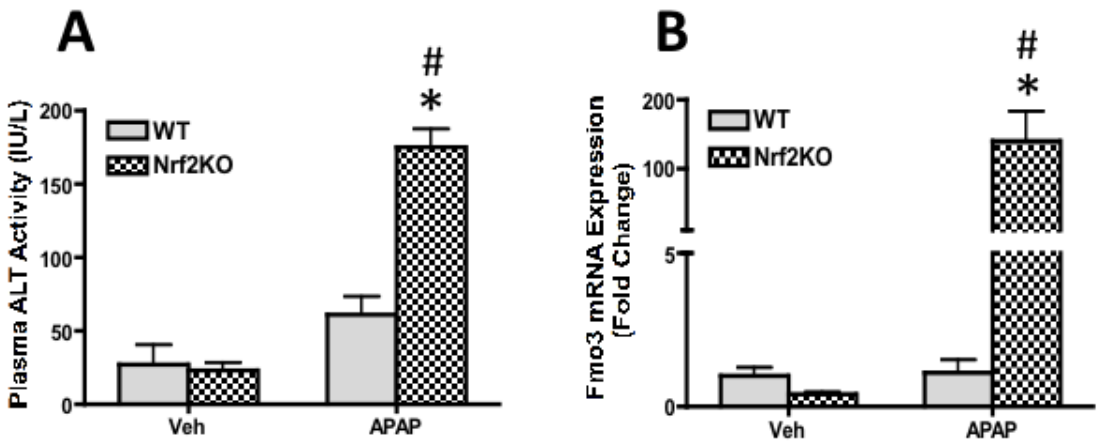


Fig. A-1.6. Plasma ALT activity and quantitative RT-PCR analysis of liver Fmo3 transcripts following a single dose APAP treatment in wild-type and Nrf2-knockout mice. Plasma and livers were collected from mice 72 h following APAP (400 mg/kg) or vehicle treatment. (A) The data are presented as mean plasma ALT (IU/L) ± SE. (B) RNA was isolated from livers and cDNA samples were analyzed by quantitative RT-PCR using Fmo3 mouse-specific primers. Gene expression was normalized to the housekeeping gene β-actin. Fmo3 mRNA expression are presented as mean Fold Change ± SE. One-way ANOVA was performed followed by the Dunnett's post-test. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle-treated group and APAP-treated group and pound (#) represent a statistical difference ($p<0.05$) compared with APAP-treated wild-type mice.

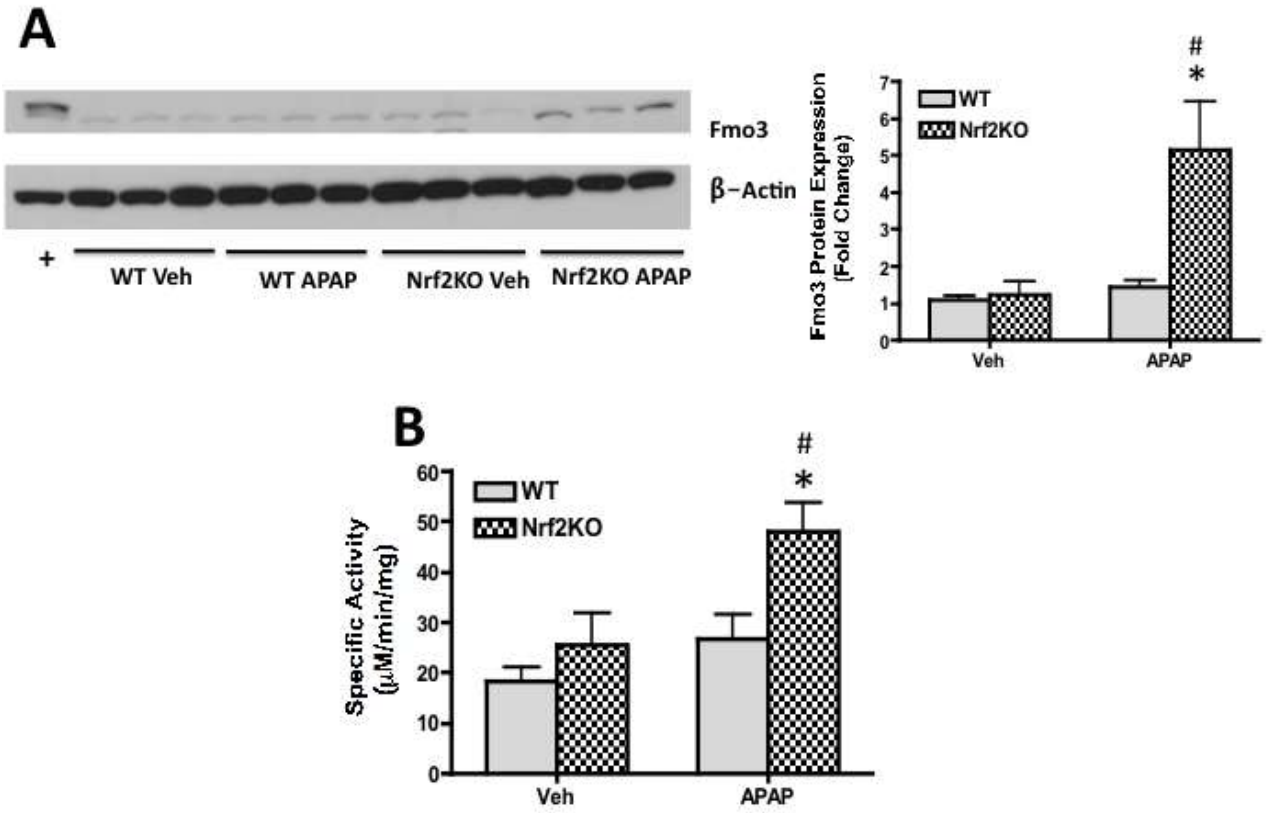

A
Fmo3
β-Actin
+
WT Veh
WT APAP
Nrf2KO Veh
Nrf2KO APAP
Fmo3 Protein Expression (Fold Change)
WT
Nrf2KO
#
*
Veh
APAP
B
Specific Activity (μM/min/mg)
WT
Nrf2KO
#
*
Veh
APAP

Fig. A-1.7. Analysis of liver Fmo3 protein expression following a single dose APAP treatment in wild-type and Nrf2-knockout mice by western blotting and enzyme activity assay. After overnight fasting, groups of wild-type and Nrf2 knock-out mice received a single dose of 400 mg/kg APAP or vehicle. Livers were collected 72 h following APAP or vehicle treatments. Western blots for Fmo3 was performed using liver microsomes from control and APAP-treated mice. Equal protein loading (10 μg protein/lane) was confirmed by detection of β-actin. Microsomal proteins isolated from naïve female mouse liver were used as a positive control indicated by "+" sign. The data are presented as blots and as mean Fmo3 protein expression (Fold Change) ± SE (A). FMO activity was measured in liver microsomes from control and APAP-treated mice using methimazole as substrate. Data are presented as mean Specific Activity (μM/min/mg) ± SE (B). Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle-treated group and APAP-treated group and pound (#) represent a statistical difference ($p<0.05$) compared with APAP-treated wild-type mice.

APPENDIX 2: CORRELATION BETWEEN CONJUGATED BISPHENOL A CONCENTRATIONS AND EFFLUX TRANSPORTER EXPRESSION IN HUMAN FETAL LIVERS

Jamie E Moscovitz[a], Muna S Nahar[b], Stuart L Shalat[c,d,e], Angela L Slitt[f], Dana C Dolinoy[b,g], Lauren M Aleksunes[a,e]

[a] Department of Pharmacology and Toxicology, Rutgers University Ernest Mario School of Pharmacy, Piscataway, NJ

[b] Department of Environmental Health Sciences, University of Michigan, Ann Arbor, MI

[c] Division of Environmental Health, School of Public Health, Georgia State University, Atlanta, Georgia

[d] Robert Wood Johnson Medical School, Rutgers University, Piscataway, NJ

[e] Environmental and Occupational Health Sciences Institute, Piscataway, NJ

[f] Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI

[g] Department of Nutritional Sciences, University of Michigan, Ann Arbor, MI

A-2.1 Abstract

Due to its widespread use in the manufacturing of consumer products over several decades, human exposure to bisphenol A (BPA) has been pervasive. Fetuses are particularly sensitive to BPA exposure, with a number of negative developmental and reproductive outcomes observed in rodent perinatal models. Xenobiotic transporters are one mechanism to extrude conjugated and unconjugated BPA from liver. In this study, the mRNA expression of xenobiotic transporters and relationships with total, conjugated, and free BPA levels were explored utilizing human fetal liver samples. The mRNA expression of BCRP and MRP4, as well as BCRP and MDR1 exhibited the highest degree of correlation, with r-squared values of 0.941 ($p<0.001$) and 0.816 ($p<0.001$), respectively. Increasing concentrations of conjugated BPA significantly correlated with high expression of MRP1 ($p<0.001$), MRP2 ($p<0.05$), and MRP3 ($p<0.05$) transporters, in addition to the transcription factor NRF2 ($p<0.001$) and its prototypical target gene, NQO1 ($p<0.001$). These data demonstrate that xenobiotic transporters may be coordinately expressed in the human fetal liver. This is also the first report of a relationship between environmentally-relevant fetal BPA levels and differences in the expression of transporters that can excrete the parent compound and its metabolites.

Abbreviations:

B2M, Beta-2-microglobulin; BCRP, breast cancer resistance protein; BPA, bisphenol A; LOQ, limit of quantification; NRF2, NF-E2-related factor 2; MDR, multidrug resistance transporter; MRP, multidrug resistance-associated transporter; NQO1, NADPH quinone oxidoreductase 1; PXR, pregnane x receptor; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase

A-2.2 Introduction

Bisphenol A (BPA) is an endocrine-disrupting chemical (EDC) used in the manufacturing of plastics and epoxy resins, and is incorporated into a variety of consumer products including food packaging, children's toys, plastic containers and medical supplies. There are multiple routes of human exposure to BPA. Leaching of BPA from consumer products has been shown to contaminate food, water, air and dust (Vandenberg et al., 2007). BPA was investigated for use as a commercial, synthetic estrogen, though it was found to have a significantly weaker potency than diethylstilbestrol (Dodds and Lawson, 1936). Because of widespread use of BPA and its endocrine-disrupting properties, there has been ongoing attention placed on the potential for BPA to negatively impact human health (Ramakrishnan et al., 2009).

Pregnant women and their offspring have been identified as potentially sensitive populations to a number of environmental EDCs, including BPA (reviewed in Rubin, 2011). Biomonitoring studies have not only detected at least one form of BPA in the serum of pregnant women, but also in a multitude of reproductive tissues including cord blood, placenta, amniotic fluid (Vandenberg et al., 2010), and fetal tissues, such as the liver (Zhang et al., 2011a; Cao et al., 2012; Nahar et al., 2013). Numerous studies conducted in rodents have associated negative outcomes with *in utero* exposure to BPA, including alterations to reproductive, neurological, behavioral, and metabolic development (reviewed in Rubin, 2011). It has been suggested that BPA may interact with multiple hormone and nuclear receptors at low doses, representing one mechanism of action for its endocrine-disrupting activity (Vandenberg et al., 2013). BPA has also been shown to alter the expression of transcription factors *in vitro*, including Pregnane X Receptor (PXR) and NF-E2-related factor 2 (NRF2), though at concentrations higher

than those observed with human environmental exposures (Takeshita et al., 2001; Sui et al., 2012; Chepelev et al., 2013).

Due to the relatively low expression of xenobiotic metabolizing enzymes and transporters, fetal livers, across species, have a reduced capacity to deactivate and excrete environmental chemicals (reviewed in Moscovitz and Aleksunes, 2013; Huse et al., 2015). In the human fetal liver, this trend corresponds with nominal expression of basal transcriptional regulators including the nuclear hormone receptors constitutive androstane receptor and PXR (Miki et al., 2005; Pascussi et al., 2007; de Sousa Abreu et al., 2009). While expression of peroxisome proliferator-activated receptors is comparable between human fetal and adult livers, raw cross threshold (CT) values are high in both groups (Abbott et al., 2010). We have shown that human fetal livers express mRNAs of the phase II detoxifying enzymes UDP-glucuronosyltransferase, (UGT) 2B15, and sulfotransferase (SULT) 1A1, albeit at levels lower than adult livers (Nahar et al., 2013). These enzymes are responsible for the conjugation of BPA to glucuronide and sulfate metabolites, respectively, which render the compound inactive (Nishiyama et al., 2002; Hanioka et al., 2008). Interestingly, it has been shown that BPA-glucuronide is transported across the placenta to the rat fetus where it can be deconjugated and thereby reactivated (Nishikawa et al., 2010). Similarly, it has been shown in sheep that the fetoplacental unit retains conjugated BPA metabolites (Corbel et al., 2013), creating a higher exposure of the fetus to bioactive BPA through conjugation-deconjugation cycling (Corbel et al., 2015; Gauderat et al., 2015). We have demonstrated that human fetal livers exhibit a wide range of quantifiable concentrations of both free and conjugated BPA, while levels in adult livers were typically below the limit of quantification (LOQ) (Nahar et al., 2013).

Efflux transporters participate in the excretion of conjugated and unconjugated forms of BPA. BPA-glucuronide is transported in the rat placenta by the multidrug resistance-associated transporter (Mrp) 1, and in the rat liver by Mrp2 (Inoue et al., 2005; Nishikawa et al., 2010). An additional report determined that the parent compound can be transported by breast cancer resistance protein (BCRP) in MDCK-II canine kidney cells (Dankers et al., 2013). Some studies have suggested differences in substrate specificity between species, and that BPA is more likely a substrate for human MRP2 and 3, in addition to the BCRP transporter (Mazur et al., 2012). A primary route of efflux has not been determined for BPA and its metabolites in humans, though in general MRP1, 2 and 3, as well as BCRP have a preference for glucuronidated and sulfated compounds (reviewed in Klaassen and Aleksunes, 2010).

While the ontogeny of human hepatic transporter expression has been described (van Kalken et al., 1992; Chen et al., 2005; Fakhoury et al., 2009; Sharma et al., 2013), modulation of expression in the presence of varying concentrations of BPA as a result of environmental exposures has not been explored. The objective of this study was to investigate relationships between concentrations of unconjugated and conjugated BPA species in human fetal livers and the role of efflux transporter expression in their disposition.

A-2.3 Materials and methods

Tissue samples

Human fetal liver samples (gestational days 74-89 n=7, 90-105 n=30, 106-120 n=12 including male n=26, female n=23) were obtained from the NIH-funded University of Washington Birth Defects Research Laboratory fetal tissue bank (2R24HD000836-47). Following consent, healthy tissue specimens were collected from women undergoing elective pregnancy terminations in the 1^{st} or 2^{nd} trimester of pregnancy. All human sample collections were carried out in accordance with the Declaration of Helsinki. Samples were immediately flash frozen and stored in polycarbonate-free tubing at -80ºC. Apart from fetal gestational age and sex, no identifying clinical data including race and ethnicity were available. Therefore, samples met the criteria for IRB exemption for human subject research (UM IRB exemption: HUM00024929).

BPA quantification

BPA concentrations were determined using high-performance liquid chromatography (HPLC) coupled with API 2000 electrospray triple-quadrupole mass spectrometer (ESI-MS/MS) by the Kannan Laboratory at the Wadsworth Center (New York State Department of Health), as previously described (Nahar et al., 2013). Several quality assurance and quality control measurements were taken to assure the validity of the HPLC ESI-MS/MS method. Each time samples were run, sample matrices were spiked with BPA standards (5ng, internal standard) and passed through the entire analytical procedure. Results indicated an average recovery of 104% (90–120%) for spiked BPA and 85% (65–120%) for spiked ^{13}C12-BPA. An external calibration curve was prepared by injecting standards at varying concentrations (10 µL of 0.05–100 ng/mL), resulting in a calibration coefficient >0.99. A procedural blank (water) was included in between every 10 samples analyzed. Concentrations of total, conjugated, and free BPA ranged from

below the LOQ at 0.071 ng/g (LOQ/√2, where LOQ=0.1 ng/g) to 96.8 ng/g (total), 49.5 ng/g (conjugated), and 50.5 ng/g (free) (Nahar et al., 2013).

RNA isolation and quantitative PCR

Total RNA was isolated from frozen liver tissue using the AllPrep DNA/RNA/Protein kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Approximately 10 to 20 mg of homogenized tissue was added to 600 mL of Buffer RLT with 1% β-mercaptoethanol in a 2 mL round bottom polypropylene tube with a 5 mm stainless steel bead. Samples were further homogenized in solution for 2 min at 20 Hz in the TissueLyser II (Qiagen). The concentration and purity of RNA was assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA (cDNA) was generated using High Capacity cDNA Synthesis (Applied Biosystems, Foster City, CA). mRNA expression was determined by quantitative PCR (qPCR) using SYBR Green-based method (Applied Biosystems) for detection of amplified products. qPCR primer sequences are listed (Table A-2.4). qPCR was performed in a 384-well plate format using the ViiA7 Real Time PCR machine (Life Technologies, Grand Island, NY). CT values were converted to delta CT values by adjusting to a reference gene (Beta-2-microglobulin, B2M) (Livak and Schmittgen, 2001).

Statistical analysis

Data were available for a total of 50 fetal liver samples. Statistical analysis was performed with the STATA v.14.0 statistical software (STATA Corp, College Station, Texas). A preliminary univariate analysis was carried out for all variables. One outlier subject exhibited a concentration of total BPA more than four standard deviations above the mean and was excluded from analysis. Correlation matrices for r-squared (r^2) and p-values were calculated for different species of BPA (total, conjugated and free), and

gestational age, and independently for the normalized values of the genes of interest (STATA, pwcorr). Separate linear regression models were computed for each BPA species. The final regression model included the normalized mRNA expression as the independent variable and the concentration of the specific form of BPA as the dependent variable. Statistically significant relationships are shown with *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

A-2.4 Results and Discussion

Bivariate analysis was conducted using a correlation matrix to look at the association between different species of BPA, and gestational age (Table A-2.1). Total BPA and free BPA levels were highly associated, with an r^2 value of 0.963 ($p<0.001$). The multivariate linear regression model was not significantly impacted by the inclusion of gestational age. Thus, this variable was excluded from the final linear regression analyses.

Strength of association was determined for the normalized mRNA expression values of each transporter (Table A-2.2). Evaluation of relationships between transporters in the fetal liver revealed the strongest correlations in mRNA expression between BCRP and MRP4, as well as between BCRP and MDR1. BCRP and MDR1 both localize to the apical surface of hepatocytes and excrete a variety of substrates into bile. MRP4 is a basolateral efflux transporter, and is known to pump sulfated conjugates back into the blood. In fact, BCRP and MRP4 have significant overlap in preference for sulfated substrates (Suzuki et al., 2003; Zelcer et al., 2003). It has been demonstrated that these two transporters can work in concert to efflux purine analogues from the livers of mice (Takenaka et al., 2007). Similarly, the cooperation between Mdr1 and Bcrp, and to a lesser extent, Mrp4, has been noted in mice at the blood-brain and blood-testis barriers (Kodaira et al., 2010).

Strength of association between normalized mRNA levels and BPA was computed by simple linear regression analysis (Table A-2.3). Increasing concentrations of conjugated BPA in fetal livers, but not total or free BPA levels, were significantly associated with elevated expression of NRF2, NADPH quinone oxidoreductase 1 (NQO1), and MRP1 mRNAs, as well as MRP2 and MRP3 mRNAs ($p<0.05$). For the three genes (NRF2, NQO1, MRP1) that were highly associated with conjugated BPA ($p<0.001$), the observed

data points, the regression line and the 95% confidence intervals were plotted (Fig. A-2.1). Up-regulation of transporters in the presence of increasing conjugated BPA levels may reflect an adaptation to enhance excretion of the parent compound and its conjugates from the fetal liver. Likewise, NRF2 has been shown to induce gene expression of MRP2 and MRP3 in human primary hepatocytes and human hepatoma HepG2 cells (Jigorel et al., 2006; Adachi et al., 2007). Interestingly, in the same study, MRP1 mRNA expression was enhanced with treatment of an NRF2 agonist, tert-butylhydroquinone, however its elevated expression was not attenuated by transfection with NRF2 siRNA (Adachi et al., 2007). In this study, a strong relationship between NQO1 and MRP1 gene expression was revealed (r^2: 0.859, Table A-2.2). Thus, while MRP1 expression may not depend on NRF2 activation, it could be induced by the presence of similar xenobiotics.

The human fetal liver is the main site of hematopoiesis from gestation day 60 to 195. This activity precedes metabolic functions the liver acquires later in development, when primary hematopoiesis shifts to the bone marrow. Thus, the structure of the fetal liver is much more loosely defined than the complex liver lobules of the adult. In addition, immunohistochemical staining showed expression of SULT1A1 protein in not only hepatocytes, but also hematopoietic cells of the developing liver (Richard et al., 2001). It is essential to note that while both MRP1 and MRP4 are lowly expressed in the human adult liver (Hilgendorf et al., 2007), data from this study indicates their raw CT values are comparable to those of other highly enriched transporters such as MRP3 and BCRP in the human fetal liver. Importantly, the presence of hematopoietic progenitors, not just hepatocytes, as well as the ontogenic expression of transporters may contribute to the altered response of the fetal liver to xenobiotics such as BPA.

Due to limited availability of samples, many ontogenic studies, particularly in humans, rely on mRNA levels to evaluate fetal expression patterns for metabolic enzymes and transporters. Studies have shown that mRNA abundance can explain up to 40% of variance in protein expression in humans, though it still cannot account for translational modifications and protein degradation that contribute to the ultimate presence of a protein in a tissue (de Sousa Abreu et al., 2009; Ramakrishnan et al., 2009). Future analysis would benefit from the quantification of efflux transporter proteins and BPA metabolites by LC/MS in addition to mRNA profiling.

Though conjugated species were differentiated from total and free BPA in these samples, the type of conjugation was not identified. As previously mentioned, while many of these transporters show preference for sulfated conjugates, they are also capable of transporting the glucuronidated and parent forms. Therefore, the unique influence of each type of conjugation pathway on xenobiotic transporter expression in fetal livers cannot be determined. However, studies in sheep have demonstrated that maternal intravenous administration of BPA results in a substantial presence of BPA-glucuronide in the fetus (Gauderat et al., 2015), and to a greater extent than BPA-sulfate (Corbel et al., 2013; Corbel et al., 2015). The authors also showed that microsomes and cytosols prepared from both fetal ewe and human livers have similar activity towards BPA glucuronidation and sulfation, respectively (Corbel et al., 2015). Taken together, it is possible that BPA-glucuronide represents a greater fraction of the conjugated BPA in the human fetal livers from this study.

This is the first report of an association between BPA species at environmentally-relevant exposure levels and differences in mRNA expression of xenobiotic transporters in fetal livers. Further, activation of NRF2 in these samples was indicated by up-

regulation of its prototypical target gene NQO1. As many other transcription factors are lowly expressed in the human fetal liver, NRF2 should be explored as a candidate transcription factor responsible for BPA-mediated transporter induction. Using a novel type of analysis in human fetal samples, this study suggests that expression of important excretory pathways in fetal livers may be altered when exposed to xenobiotics *in utero*.

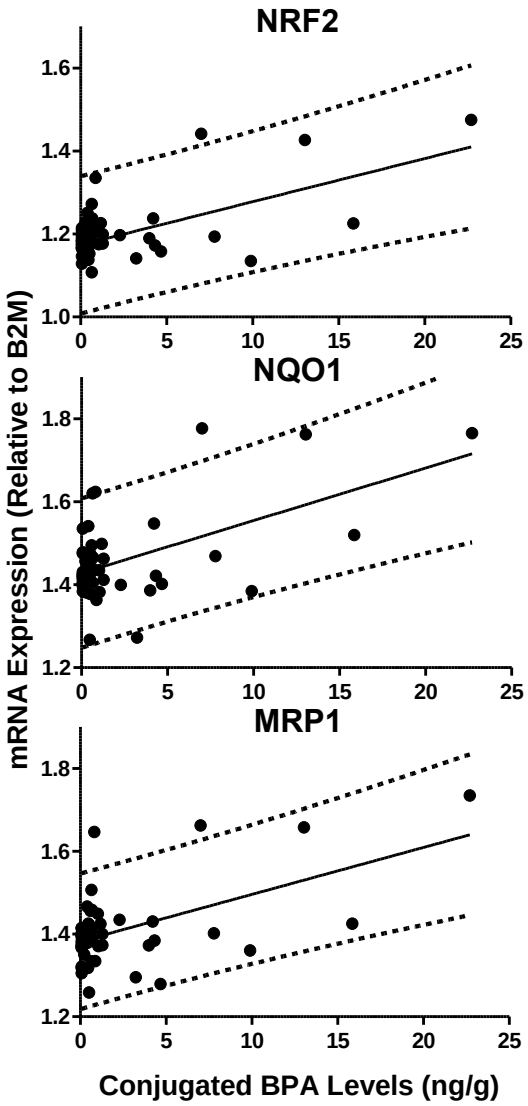


Fig. A-2.1. Univariate modeling of gene expression and conjugated BPA levels in fetal livers. Linear regressions for NRF2 mRNA, NQO1 mRNA and MRP1 mRNA, and conjugated BPA levels are shown. Data are presented as mean relative expression (n=49, normalized to B2M). Dashed lines represent 95% confidence limits.

Table A-2.1. Correlation matrix for sample characteristics[1]

	Total BPA	Conjugated BPA	Free BPA	Gestational Age
Total BPA	1.00			
Conjugated BPA	0.748***	1.00		
Free BPA	0.963***	0.539***	1.00	
Gestational Age	0.199	0.067	0.224	1.00

[1]Summary of the correlations between different BPA species concentrations and gestational age. A value of 1.0 represents a perfect correlation. Statistically significant relationships are shown with ***$p<0.001$.

Table A-2.2. Normalized gene expression correlation matrix[1]

	MRP1	MRP2	MRP3	MRP4	MDR1	BCRP	NRF2	NQO1
MRP1	1.00							
MRP2	0.739***	1.00						
MRP3	0.462***	0.301*	1.00					
MRP4	0.380**	0.169	0.741***	1.00				
MDR1	0.320*	0.147	0.689***	0.734***	1.00			
BCRP	0.370**	0.212	0.796***	0.941***	0.816***	1.00		
NRF2	0.418**	0.235	0.315*	0.266	0.266	0.264	1.00	
NQO1	0.859***	0.597***	0.300*	0.302*	0.167	0.233	0.475***	1.00

[1]Summary of the correlations between gene expression of different transcription factors and transporters in human fetal liver. A value of 1.0 represents a perfect correlation. Statistically significant relationships are shown with *p<0.05, **p<0.01, and ***p<0.001.

Table A-2.3. Regression coefficients for gene expression and BPA levels in human fetal livers[1]

	BPA Species					
	Total		Conjugated		Free	
	β	p	β	p	β	p
MRP1	1.45×10^{-3}	0.666	1.13×10^{-2}	<0.001***	4.36×10^{-4}	0.728
MRP2	3.79×10^{-4}	0.815	5.54×10^{-3}	0.038*	-3.14×10^{-4}	0.779
MRP3	8.82×10^{-4}	0.350	7.12×10^{-3}	0.013*	2.26×10^{-4}	0.851
MRP4	1.19×10^{-3}	0.185	4.79×10^{-3}	0.085	1.11×10^{-3}	0.332
MDR1	5.16×10^{-4}	0.677	3.37×10^{-3}	0.380	2.62×10^{-4}	0.868
BCRP	1.03×10^{-3}	0.288	4.45×10^{-3}	0.138	9.08×10^{-4}	0.462
NRF2	1.82×10^{-3}	0.057	1.04×10^{-2}	<0.001***	1.19×10^{-3}	0.334
NQO1	1.66×10^{-3}	0.124	1.27×10^{-2}	<0.001***	1.19×10^{-3}	0.334

[1]Regression coefficients (β) were calculated from simple linear regression analysis for each gene and BPA species (n=49). Statistically significant relationships are shown with *p<0.05 and ***p<0.001.

Table A-2.4. qPCR primer sequences

Primer	Forward (5' to 3')	Reverse (5' to 3')
MRP1	CATCATCCCCCAGGACCCTGTTT	CACTGAGGTTCTCCCCGCCTTC
MRP2	AGCCATGCAGTTTTCTGAGGCCT	TGGTGCCCTTGATGGTGTGC
MRP3	CTTCCTGGTGACCCTGATCACCCT	TGCTGGATCCGTTTCAGAGACACA
MRP4	TCCAGACATTGCTACAAGTGGTTGG	CTCCGAGTTGTAGATTCCAGGCGCT
MDR1	TTGAAATGAAAATGTTGTCTGG	CAAAGAAACAACGGTTCGG
BCRP	ATCAGCTGGTTATCACTGTGAGGCC	AGTGGCTTATCCTGCTTGGAAGGC
NRF2	TCCCAGCAGGACATGGATTT	TCTTCATCTAGTTGTAACTGAGCG
NQO1	CAAAGGACCCTTCCGGAGTAA	ACTTGGAAGCCACAGAAATGC
B2M[1]	TCGCTCCGTGGCCTTAGCTG	CAATGTCGGATGGATGAAACCCAG

[1]B2M was used as a reference gene to normalize expression.

APPENDIX 3: REGULATION OF DRUG DISPOSITION GENE EXPRESSION IN PREGNANT MICE WITH CAR RECEPTOR ACTIVATION

Amanda S Bright[a], Guadalupe Herrera-Garcia[b], Jamie E Moscovitz[a], Dahea You[a], Grace L Guo[a,c], Lauren M Aleksunes[a,c]

[a] Department of Pharmacology and Toxicology, Rutgers University Ernest Mario School of Pharmacy, 170 Frelinghuysen Road, Piscataway, NJ 08854, USA

[b] Department of Obstetrics and Gynecology, Rutgers-Robert Wood Johnson Medical School, 1 Robert Wood Johnson Place, New Brunswick, NJ 08901, USA

[c] Environmental and Occupational Health Sciences Institute, 170 Frelinghuysen Road, Piscataway, NJ 08854, USA

A-3.1 Abstract

More than half of pregnant women use prescription medications in order to maintain both maternal and fetal health. The constitutive androstane receptor (Car) critically affects the disposition of chemicals by regulating the transcription of genes encoding metabolic enzymes and transporters. However, the effects of Car activation on chemical disposition during pregnancy are unclear. This study aims to determine the degree to which pregnancy alters the expression of drug metabolizing enzymes and transporters in response to the pharmacological activation of Car. To test this, pregnant C57BL/6 mice were administered IP doses of vehicle, or a potent Car agonist, TCPOBOP, on gestation days 14, 15 and 16. Hepatic mRNA and protein expression of Car target genes (phase I, II and transporters) were quantified on gestation day 17. Pregnancy-related changes, such as induction of Cyp2b10, Ugt1a1 and Sult1a1 and repression of Ugt1a6, Gsta1, Gsta2 and Mrp6, were observed. Interestingly, the induction of Cyp2b10, Gsta1, Gsta2 and Mrp2-4 mRNAs by TCPOBOP was attenuated in maternal livers suggesting that Car activation is impeded by the biochemical and/or physiological changes that occur during gestation. Taken together, these findings suggest that pregnancy and pharmacological activation of Car can differentially regulate the expression of drug metabolism and transport genes.

A-3.2 Introduction

Pharmacotherapy is necessary to treat new and pre-existing conditions during pregnancy. It is estimated that 64% of women use prescription drugs (excluding vitamins and minerals) during pregnancy (Andrade et al., 2004). There are a number of adaptations in the mother that occur to support the needs of the growing fetus (Dai et al., 2011). The maternal liver undergoes significant physiological and biochemical changes that, in turn, alter the disposition and metabolism of endobiotics such as lipids, bile acids, glucose and cholesterol, as well as xenobiotics (Nakai et al., 2002; Jeong, 2010; Dai et al., 2011; Lindsay et al., 2015). Moreover, the expansion of plasma volume during pregnancy contributes to elevations in cardiac output and increased hepatic blood flow, which can enhance the clearance of high extraction ratio drugs (Nakai et al., 2002). Despite the need for pharmacotherapy, little data exist regarding the safety and appropriate dosing regimens of drugs due to ethical concerns that preclude the participation of pregnant women in randomized clinical trials.

The constitutive androstane receptor (Human CAR/rodent Car, NR1I3) is a nuclear receptor that is highly enriched in the liver (Petrick and Klaassen, 2007). As its name suggests, in either the absence, but particularly in the presence of an agonist, Car heterodimerizes with the retinoid X receptor alpha and binds to cognate response elements of target genes, thereby up-regulating their transcription (reviewed in Tolson and Wang, 2010). Chemical activators of Car are structurally diverse and include drugs such as phenobarbital, the fungicide propiconazole, as well as synthetic compounds such as 1,4-bis(2-(3,5-dichloropyridyloxy)) benzene (TCPOBOP) (Oshida et al., 2015).

Car is considered a master regulator of hepatic drug disposition because downstream targets include a number of drug metabolizing enzymes and xenobiotic transporters.

Activation of CAR/Car in the liver is routinely monitored by the up-regulation of the human cytochrome P450 enzyme, CYP2B6, or the mouse ortholog Cyp2b10 (Honkakoski et al., 1998; Kawamoto et al., 1999). In addition, rodent Car has been shown to regulate several phase II conjugating enzymes, including members of the UDP-glucuronosyltransferase (Ugt), sulfotransferase (Sult) and glutathione S-transferase (Gst) families (Maglich et al., 2004; Buckley and Klaassen, 2009; Tolson and Wang, 2010; Aleksunes and Klaassen, 2012). Ligand-activated Car has also been shown to alter the expression of hepatic uptake transporters including the organic anion transporter proteins (Oatps) (Guo et al., 2002; Cheng et al., 2005) and efflux transporters such as the multidrug resistance-associated proteins (Mrps) in rodents (Maher et al., 2005; Petrick and Klaassen, 2007).

Our laboratory, along with others, has previously demonstrated that the expression of nuclear receptors and their prototypical target genes are repressed in mice during pregnancy (Sweeney et al., 2006; Aleksunes and Klaassen, 2012; Aleksunes et al., 2012; Wen et al., 2013). The altered function of transcription factors in the maternal liver corresponds with variable changes in the expression and activity of phase I and phase II enzymes, and an overall down-regulation of transporters. Notably, we have observed that pregnant mice have reduced levels of hepatic Car target genes including Ugt1a6, Gsta1, and Mrp3 (Aleksunes and Klaassen, 2012; Wen et al., 2013). Early studies revealed the ability of prototypical microsomal enzyme inducers such as phenobarbital to alter xenobiotic metabolism during pregnancy (Bell et al., 1975; Vore and Soliven, 1979). Treatment of pregnant guinea pigs with phenobarbital resulted in enhanced total Cyp content (Hill and Abramson, 1990). Likewise, administration of phenobarbital induced the glucuronidation of estrone and estradiol to a greater extent in pregnant rats than in non-pregnant females (Vore and Soliven, 1979). However, these studies preceded our

knowledge of individual Cyp and Ugt isoforms that are differentially up- or down-regulated by phenobarbital. As a result, further investigation is needed into the regulation of Car targets during pregnancy using a specific and potent pharmacological agonist.

It is recognized that pregnancy differs between species with respect to gestation length, number of offspring, placentation, and other factors (reviewed in Malassine et al., 2003). Nonetheless, rodents are a useful model to assess adaptive responses to pregnancy and are routinely used in reproductive toxicology studies to assess the safety of new pharmaceuticals. Therefore, the purpose of this study was to determine to what degree pregnancy alters the expression of drug metabolizing enzymes and transporters in response to the pharmacological activation of Car in mice.

A-3.3 Materials and methods

Chemicals

All chemicals including 1,4-bis(2-(3,5-dichloropyridyloxy)) benzene (TCPOBOP) were obtained from Sigma Aldrich (St. Louis, MO), unless otherwise specified.

Animals

Adult male and female wild-type C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were allowed access to feed and water *ad libitum*. All mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal care facility in temperature-, light-, and humidity-controlled rooms. The Rutgers University Institutional Animal Care and Use Committees approved all animal studies. A subset of female mice were mated overnight with male mice and separated the following morning (designated gestation day 0). The remaining female mice were utilized as virgin controls. Virgin and pregnant time-matched mice (n=6-9) received an intraperitoneal injection of vehicle (5 ml/kg corn oil) or TCPOBOP (1 mg/kg) on gestation days 14, 15 and 16. Livers were collected, weighed, and snap frozen on gestation day 17.

RNA isolation

Total RNA was isolated from snap frozen whole livers using RNABee (Tel-Test, Friendswood, TX) and the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer recommendations. Concentrations of total RNA were quantified by a NanoDrop spectrophotometer (Fisher Scientific, Pittsburgh, PA). Complimentary DNA (cDNA) was synthesized using High Capacity cDNA Synthesis for real time (RT)-PCR (Applied Biosystems, Foster City, CA).

Quantitative PCR

Quantitative PCR was performed in 384-well plate format using the Viia7 Real Time PCR machine (Applied Biosystems, Warrington, UK). Specific forward and reverse primers are listed in Table A-3.1. SYBR Green fluorescence (Applied Biosystems) was used to detect amplified products. Ribosomal protein l13a (Rpl13a) was used as a reference gene. Ct values were converted to delta delta Ct values by comparing to Rpl13a.

Western blot analysis

Liver tissue was homogenized in 10 mM Tris base and 150 mM sucrose buffer, pH 7.5 using the TissueLyser LT Adapter (Qiagen), as per the manufacturer's protocol. Protein concentrations were determined using a BCA protein assay kit (Pierce Biotechology, Rockford, IL). Protein homogenates (50 µg) were separated on a 4-12% NUPAGE Novex Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene fluoride membrane. Western blot detection of Ugt1a1, 1a9, and Mrp2-4 and 6 was performed as described previously (Aleksunes et al., 2008b; Wen et al., 2013). Protein bands were visualized using SuperSignal West Dura Chemiluminscent Substrate (Thermo Scientific, Pittsburgh, PA) on a FluorChemE Systems Imager (Protein Simple, Santa Clara, CA). Relative band intensities were semi-quantified utilizing Alpha View SA software (Protein Simple) and normalized to a loading control beta-actin (ab8227, Abcam, Cambridge, MA).

Indirect immunofluorescence

Immunofluorescent staining was performed on liver cryosections (6 µm) as previously described (Aleksunes and Klaassen, 2012). Images were acquired on a Zeiss Observer D1 microscope at 200x magnification using a Jenoptik camera. All sections were stained

and imaged under uniform conditions for each antibody. Negative controls without primary antibody were also included (data not shown).

Data analysis

All results are expressed as mean ± standard error (SE). Data are normalized to virgin controls (set to 1.0). mRNA and protein expression were analyzed using a 2-way ANOVA or a 1-way ANOVA followed by a Newman-Keuls multiple comparison post-hoc test when appropriate using GraphPad Prism V6 (GraphPad, La Jolla, CA) software. Significance was set at $p \leq 0.05$.

A-2.4 Results and Discussion

Activation of Car in pregnant mice following TCPOBOP administration.

Pregnancy did not significantly alter Car mRNA levels, while treatment with TCPOBOP reduced its levels 45 to 50% in both virgin and pregnant mice as compared to virgin controls (Fig. A-3.1). A 50% repression of Car mRNA by TCPOBOP was similar to prior studies in non-pregnant animals (Assem et al., 2004; Petrick and Klaassen, 2007; Tolson and Wang, 2010). The strong activation of Car by TCPOBOP may initiate a negative feedback repression of its own expression to limit further signaling.

The prototypical human CAR target gene, CYP2B6, contributes to the metabolism of up to 8% of commonly used drugs, including the pain reliever methadone and sertraline, an antidepressant often prescribed in pregnancy (Vore and Soliven, 1979; Kobayashi et al., 1999; Jeong, 2010; Dickmann and Isoherranen, 2013). The mRNA expression of Cyp2b10, the mouse ortholog of CYP2B6, was enhanced by nearly 8-fold in pregnant mice, and was further induced by TCPOBOP in virgin and pregnant mice by 22- and 18.5-fold, respectively (Fig. A-3.1). There are conflicting reports regarding the adaptive regulation of Cyp2b10 mRNA during pregnancy with different reports demonstrating down-regulation (Dai et al., 2011; Koh et al., 2011; Wen et al., 2013), up-regulation (Milona et al., 2010b), or no change in its expression (Koh et al., 2011). Nonetheless, these data demonstrate that Car activation was achieved in mice treated with TCPOBOP. Likewise, enhanced liver-to-body weight ratios were observed in both virgin and pregnant mice treated with TCPOBOP (Fig. A-3.8), confirming liver enlargement typically associated with Car activation in rodents (Lin et al., 2011).

Regulation of phase II metabolizing enzymes in pregnant mice following Car activation.

Maternal livers exhibited differential changes in the expression of Ugt isoforms. Notably, pregnant mice had enhanced Ugt1a1 mRNA (35%), repressed Ugt1a6 mRNA (80%) and no change in levels of Ugt1a9 or 2b5 mRNAs compared to virgin controls (Fig. A-3.2A). Treatment with TCPOBOP enhanced expression of Ugt1a1 and 1a9 mRNAs in virgin as well as pregnant mice. Tolson *et.* al previously reported that activation of Car by phenobarbital or TCPOBOP increases the expression of Ugt1a1 and Ugt1a9, suggesting that Car may serve as a general regulator of glucuronidation enzymes (Tolson and Wang, 2010). While western blot confirmed that similar to mRNA levels, Ugt1a1 protein expression was increased by 27% in TCPOBOP-treated virgin mice, the same was not observed for Ugt1a9 (Fig. A-3.2B). However, both vehicle- and TCPOBOP-treated pregnant mice had a 15 to 20% reduction in Ugt1a1 protein levels compared to vehicle-treated virgin controls.

Compared to expression in vehicle-treated virgin controls, livers of virgin and pregnant mice treated with TCPOBOP showed reduced Ugt1a6 mRNAs. A similar down-regulation of Ugt1a6 was observed with TCPOBOP treatment in virgin and pregnant mice. The repression of Ugt1a6 mRNA by TCPOBOP was surprising given that Ugt1a6 has been shown to be up-regulated by prototypical enzyme inducers of Car in the livers of mice (Buckley and Klaassen, 2009) and rats (Shelby and Klaassen, 2006). In a similar study, Ugt1a6 expression was unaltered in male mice treated with 300 μg/kg of TCPOBOP for four days (Buckley and Klaassen, 2009). Possible explanations for the divergent responses include variation in dose, and potential gender differences in the regulation of Ugt1a6.

Gsta1 and a2 are known targets of Car activation in mouse livers (Knight et al., 2008; Aleksunes and Klaassen, 2012). Compared to vehicle-treated virgin mice, pregnancy

decreased the mRNA expression of both Gsta1 and Gsta2 by 84 and 78%, respectively (Fig. A-3.3). TCPOBOP-mediated induction of both Gst isoforms was somewhat attenuated in pregnant mice compared to virgin mice. Consistent with a previous report, Sult1a1 mRNA was increased by 55 to 75% in pregnancy (Wen et al., 2013); however administration of TCPOBOP did not further alter its expression.

Regulation of hepatic transporters in pregnant mice following Car activation.

Pregnancy did not significantly change the expression of the uptake transporters Oatp1a1 or Oatp1a4, although opposing trends for enhanced Oatp1a1 and reduced Oatp1a4 levels were observed (Fig. A-3.4). Similar to a prior study in male mice, treatment with TCPOBOP reduced hepatic mRNA expression of Oatp1a1 (Cheng et al., 2005). While this trend was observed in both virgin and pregnant TCPOBOP-treated mice, Oatp1a1 was significantly repressed only in TCPOBOP-treated pregnant mice, as compared to vehicle-treated counterparts.

Our laboratory has previously demonstrated that Mrps, a group of efflux transporters, are differentially regulated in the maternal liver during pregnancy. Specifically, repression of Mrp2, 3 and 6 and induction of Mrp4 mRNAs were observed in pregnant mice (Aleksunes and Klaassen, 2012). In the present study, we observed similar pregnancy-related trends in vehicle-treated pregnant mice with the most notable change being the down-regulation of Mrp6 by 50% (Fig. A-3.5). As expected, TCPOBOP treatment in virgin mice up-regulated the mRNA expression of Mrp2 (0.76-fold), Mrp3 (0.68-fold), and Mrp4 (18-fold) (Maher et al., 2005; Petrick and Klaassen, 2007). Likewise, TCPOBOP increased Mrp2-4 mRNAs in pregnant mice, though the magnitude of Mrp3 and 4 inductions were less than those observed in virgin mice. At the protein level, the up-regulation of Mrp2-4 proteins by TCPOBOP was also attenuated in pregnant mice

compared to virgin mice (Fig. A-3.6). For example, Mrp2 protein was induced 2.6-fold in virgin mice treated with TCPOBOP, but only 1.6-fold in pregnant mice. It has been shown previously that reproductive hormones can mediate the internalization of transporters on the canalicular membrane of hepatocytes. Of importance to this study, Mrp2 localization can be disrupted by a metabolite of estradiol, estradiol-17β-D-glucuronide (Mottino et al., 2005). Indirect immunofluorescence confirmed the apical localization of Mrp2 to the canalicular membrane of hepatocytes (Fig. A-3.7). The intensity of staining of Mrp2 in liver sections was enhanced by TCPOBOP treatment in both virgin and pregnant mice; however, the overall intensity was lower in sections from pregnant mice.

Compared to Mrp2-4, the regulation of Mrp6 expression by Car is quite different. A prior study in male mice has demonstrated the ability of Car activation by phenobarbital and TCPOBOP to induce Mrp6 mRNAs (Maher et al., 2005). Interestingly, we observed the opposite in both virgin and pregnant mice treated with TCPOBOP. Similar to the down-regulation of Mrp6 observed in vehicle-treated pregnant mice, the administration of TCPOBOP also led to reduced Mrp6 mRNA expression by 45 and 58% in virgin and pregnant mice, respectively (Fig. A-3.5). Consistent with the down-regulation of mRNA expression in pregnant mice, hepatic Mrp6 protein expression was reduced by 35-50%. Both virgin and pregnant TCPOBOP-treated mice showed comparable down-regulation of mRNA and protein expression (Fig. A-3.6). Indirect immunofluorescence revealed localization of Mrp6 to the sinusoidal membrane of hepatocytes that was uniform throughout the lobule (Fig. A-3.7). Compared to vehicle-treated virgin controls, the intensity of staining was lower in livers from TCPOBOP-treated virgin mice and even further reduced in both vehicle- and TCPOBOP-treated pregnant mice. The mechanism(s) underlying the down-regulation of Mrp6 during pregnancy and lack of

response to TCPOBOP is unclear. In comparison to other hepatic Mrp transporters, Mrp6 has fewer identified substrates (Smith et al., 1998; Belinsky et al., 2002; Ilias et al., 2002). Additionally, whereas Mrp2-5 were shown to be induced in altered physiological states, such as models of extrahepatic cholestasis and type I diabetes, Mrp6 was unchanged in mice (Slitt et al., 2007; Aleksunes et al., 2013). It is likely that Mrp6 may not be an important compensatory pathway for basolateral excretion of endo- and xenobiotics, unlike its counterparts Mrp3 and Mrp4.

In conclusion, the current study assessed the responsiveness of the mouse liver to Car activation by the specific and potent agonist, TCPOBOP, during pregnancy. Interestingly, the ability of TCPOBOP to induce known Car targets including Cyp2b10, Gsta1, Gsta2 and Mrp2-4 was impaired during pregnancy because for each of these genes, the maximal induction following TCPOBOP was lower in pregnant mice compared to virgin mice. These findings suggest that pregnancy impedes activation of Car in maternal mouse livers. Prior work showed that induction of microsomal estrone and estradiol glucuronyltransferase activity with phenobarbital was similar between non-pregnant and pregnant rabbits (Vore and Soliven, 1979). Although when adjusted for total liver size, greater microsomal activity was observed in livers from pregnant rabbits treated with phenobarbital compared to non-pregnant controls (Vore and Soliven, 1979). In our current study, Ugt1a1 and 1a9 proteins were not enhanced in pregnant mice treated with TCPOBOP, which may reflect differences in species, experimental endpoint and/or selection of Car activator. It is not clear whether the differences in Car responsiveness during pregnancy are due to changes in the pharmacokinetics of TCPOBOP or result from altered responsiveness of Car receptor activation. The induction of Cyp2b10 mRNA in vehicle-treated pregnant mice was not anticipated given the overall reduced signaling of Car-related targets and a trend for reduced Car mRNA

itself (Fig. A-3.1). It should be noted that the translocation of Car protein was not quantified which may reveal additional insight into signaling pathways activated by pregnancy.

Future studies will address the ability of pregnancy-related hormones, such as estradiol, glucocorticoids, growth hormones, and progesterone, to influence Car function. Circulating concentrations of 17ß-estradiol steadily increase from gestation days 11 to 17 in pregnant mice (Aleksunes et al., 2012). Koh *et al.* have demonstrated that pregnancy-relevant concentrations of estradiol (> 100 nM) can activate Car and estrogen receptor/AP-1 leading to the transactivation of the CYP2B6 gene in rat and human hepatocytes (Koh et al., 2012). Moreover, the ability of exogenous estrogens (17ß-estradiol and estrone) to modulate mouse Car function through the NR1 enhancer region of the *Cyp2b* promoter has been demonstrated *in vitro* using primary hepatocytes and estradiol-treated mice (Kawamoto et al., 2000). Taken together, these data support the up-regulation of Cyp2b10 mRNA in vehicle-treated pregnant mice. In addition, further work is necessary to determine whether a similar attenuated response to CAR activation is observed in human livers during pregnancy. Despite the reduced signaling observed in maternal mouse livers, these findings suggest that the altered expression profiles of certain Car target genes as a result of pregnancy can be partially reversed by pharmacological intervention.

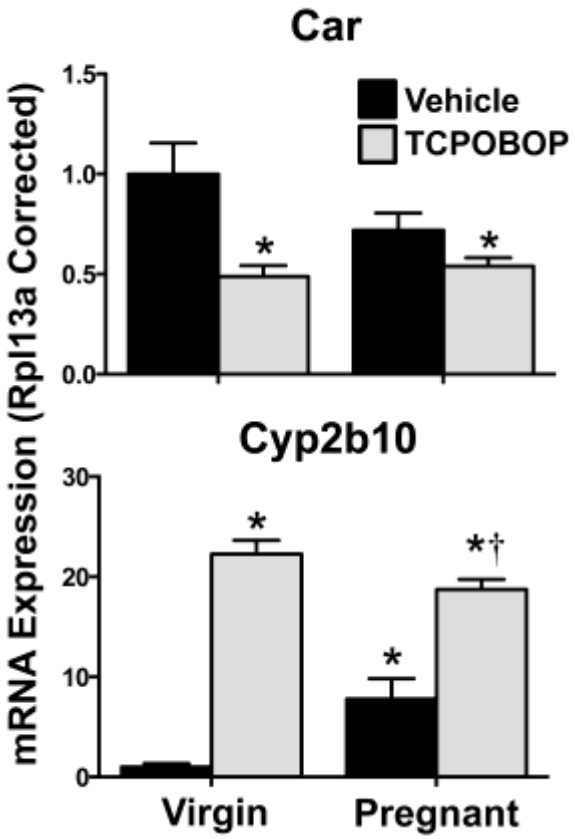


Fig. A-3.1. Expression of Car and target gene Cyp2b10 in pregnant mice treated with TCPOBOP. Hepatic mRNA expression of Car and its target gene Cyp2b10 was quantified in virgin and pregnant mice treated with vehicle or TCPOBOP. Data were normalized to vehicle-treated virgin controls (set to 1.0) and presented as mean relative expression ± SE (n=6-9). Black bars represent vehicle-treated mice, and light grey bars represent TCPOBOP-treated mice. Asterisks (*) represent differences ($p \leq 0.05$) compared with vehicle-treated virgin mice. Daggers (†) represent differences ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

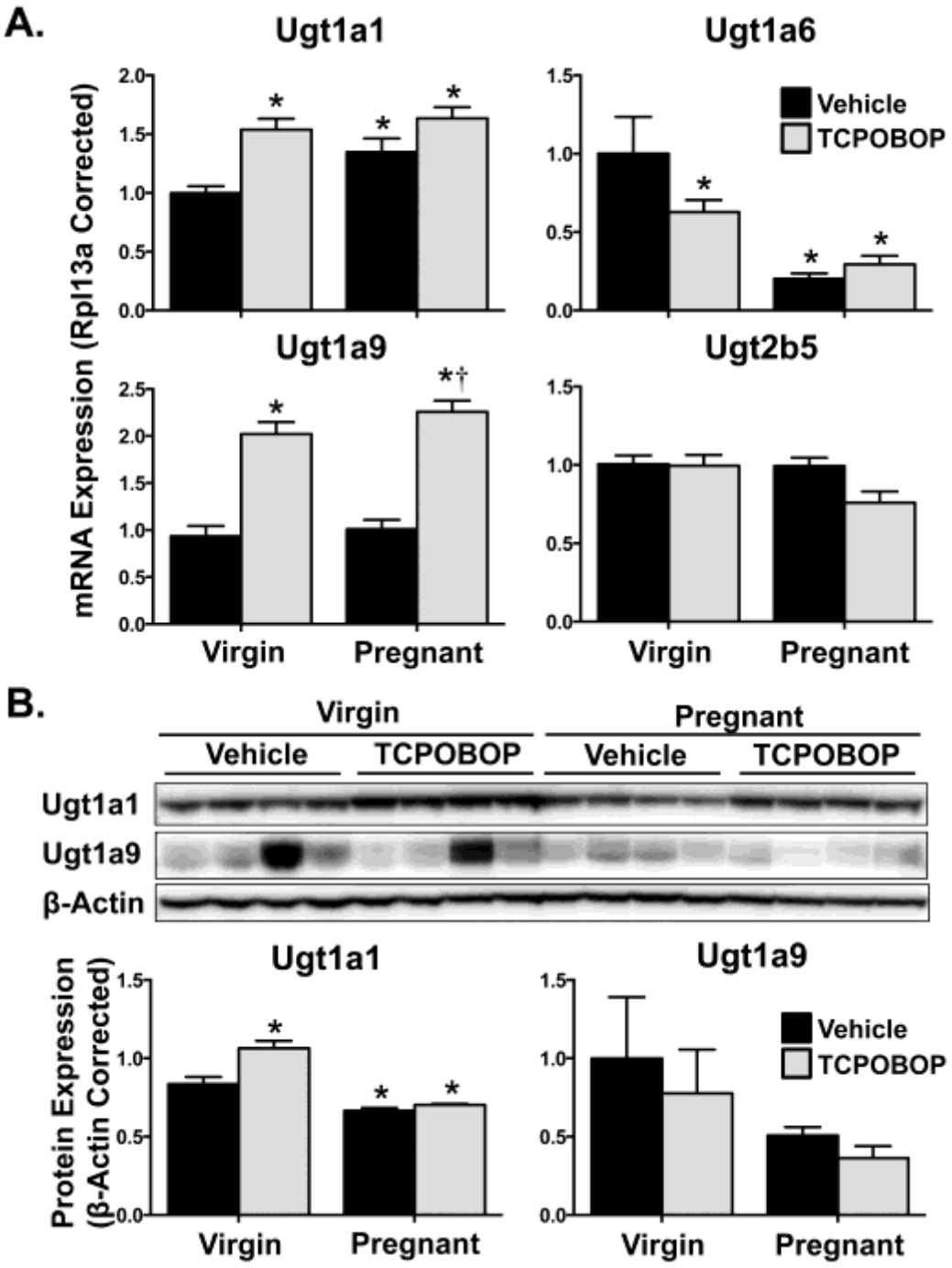

A.
Ugt1a1
Ugt1a6
Ugt1a9
Ugt2b5
mRNA Expression (Rpl13a Corrected)
Vehicle
TCPOBOP
Virgin
Pregnant
B.
Virgin
Pregnant
Vehicle
TCPOBOP
Ugt1a1
Ugt1a9
β-Actin
Protein Expression (β-Actin Corrected)

Fig. A-3.2. Expression of Ugt enzymes in pregnant mice treated with TCPOBOP. Hepatic (A) mRNA and (B) protein expression of Ugt enzymes was quantified in virgin and pregnant mice treated with vehicle or TCPOBOP. Representative western blots are shown and semi-quantification presented below. Data were normalized to virgin vehicle-treated controls (set to 1.0) and presented as mean relative expression ± SE (n=6-9). Black bars represent vehicle-treated mice, and light grey bars represent TCPOBOP-treated mice. Asterisks (*) represent differences ($p \leq 0.05$) compared with vehicle-treated virgin mice. Daggers (†) represent differences ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

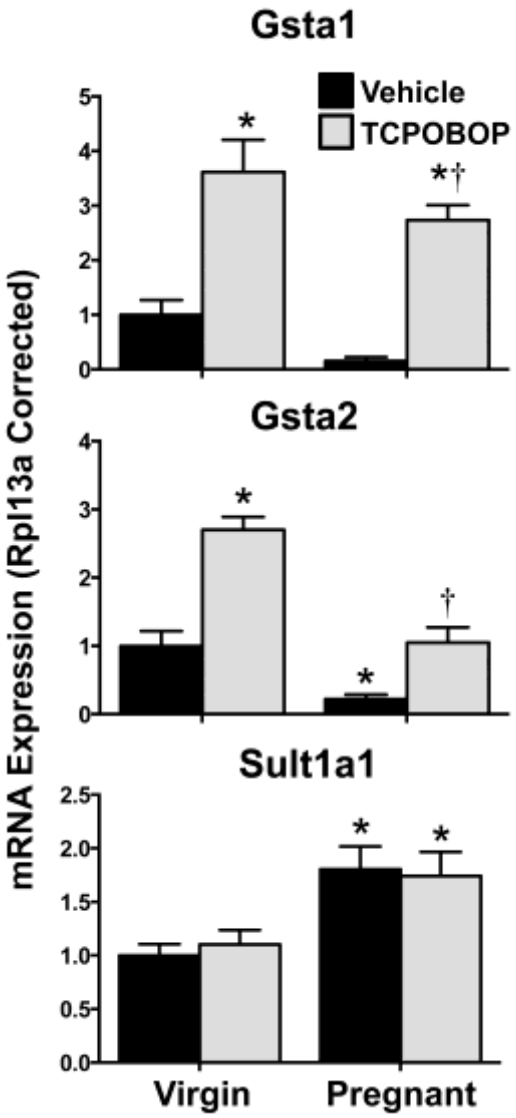


Fig. A-3.3. Expression of Sult and Gst enzymes in pregnant mice treated with TCPOBOP. Hepatic mRNA expression of phase II metabolic enzymes was quantified in virgin and pregnant mice treated with vehicle or TCPOBOP. Data were normalized to vehicle-treated virgin controls (set to 1.0) and presented as mean relative expression ± SE (n=6-9). Black bars represent vehicle-treated mice, and light grey bars represent TCPOBOP-treated mice. Asterisks (*) represent differences (p≤0.05) compared with vehicle-treated virgin mice. Daggers (†) represent differences (p≤0.05) compared with vehicle-treated pregnant mice.

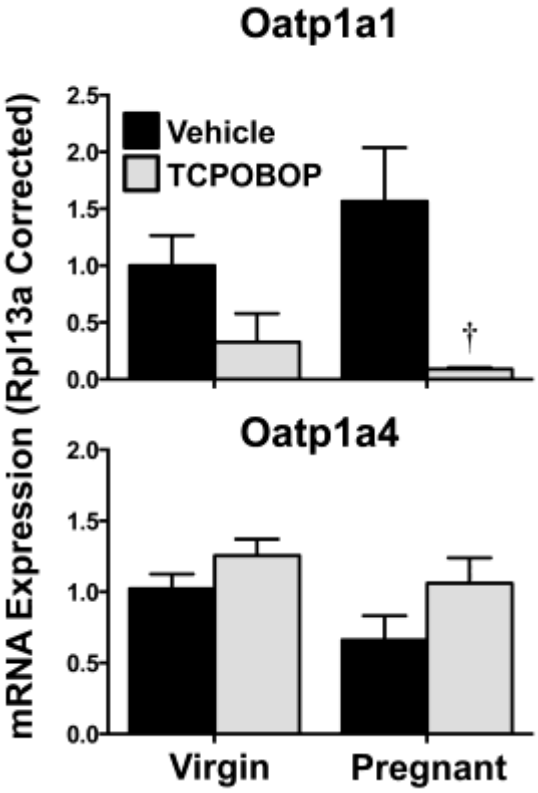


Fig. A-3.4. Expression of uptake transporter genes in pregnant mice treated with TCPOBOP. Hepatic mRNA expression of uptake transporters Oatp1a1 and Oatp1a4 was quantified in virgin and pregnant mice treated with vehicle or TCPOBOP. Data were normalized to vehicle-treated virgin controls (set to 1.0) and presented as mean relative expression ± SE (n=6-9). Black bars represent vehicle-treated mice, and light grey bars represent TCPOBOP-treated mice. Daggers (†) represent differences ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

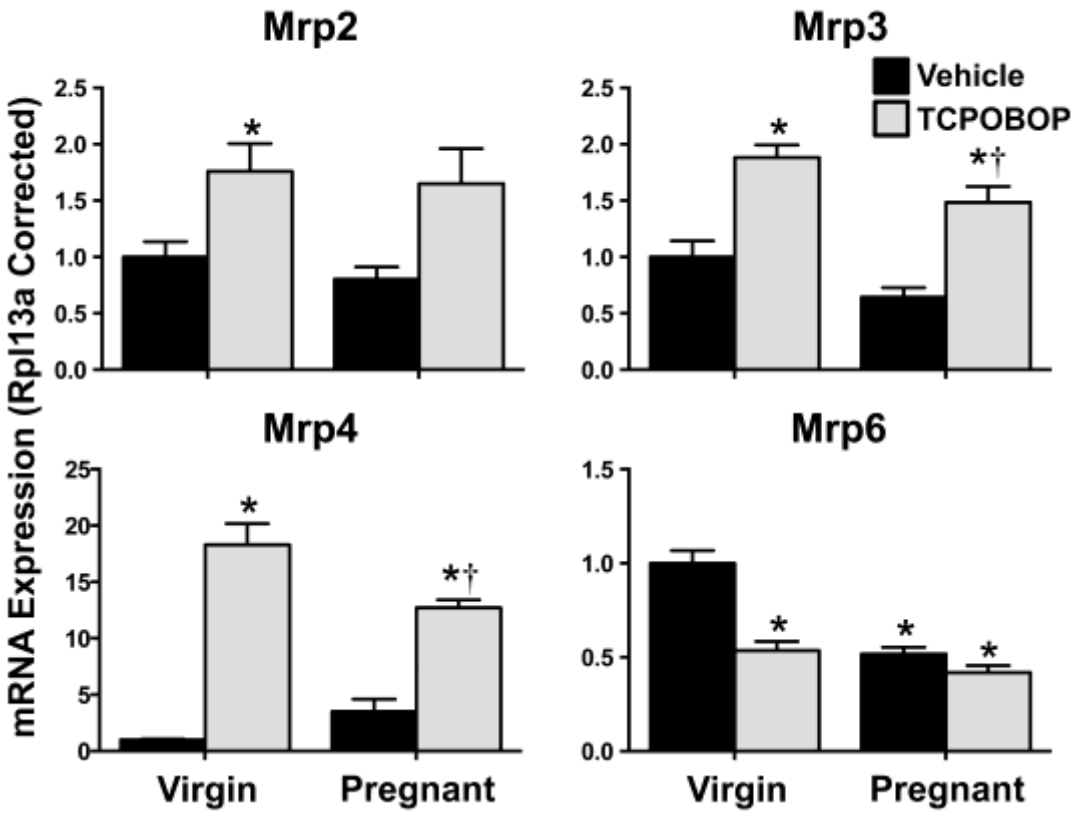


Fig. A-3.5. Expression of efflux transporter genes in pregnant mice treated with TCPOBOP. Hepatic mRNA expression of efflux transporters was quantified in virgin and pregnant mice treated with vehicle or TCPOBOP. Data were normalized to virgin wild-type controls (set to 1.0) and presented as mean relative expression ± SE (n=6-9). Black bars represent vehicle-treated mice, and light grey bars represent TCPOBOP-treated mice. Asterisks (*) represent differences (p≤0.05) compared with vehicle-treated virgin mice. Daggers (†) represent differences (p≤0.05) compared with vehicle-treated pregnant mice.

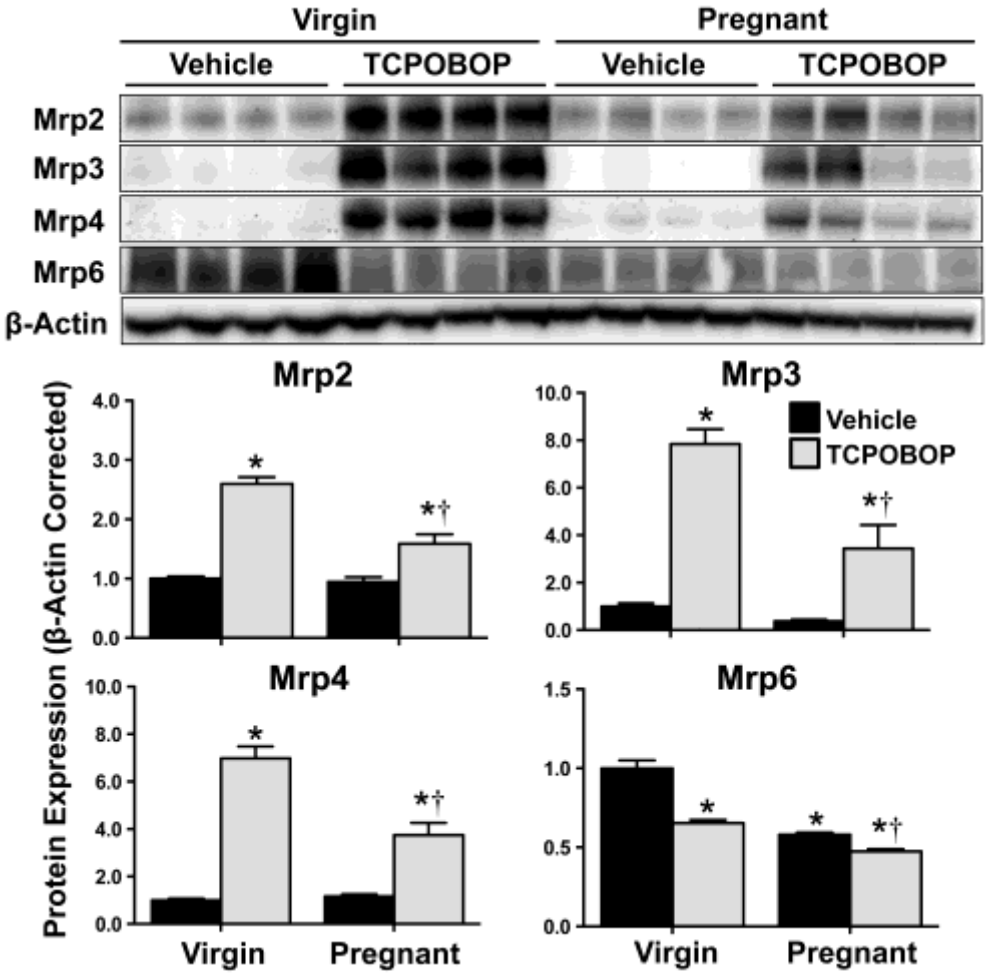


Fig. A-3.6. Expression of efflux transporter proteins in pregnant mice treated with TCPOBOP. Protein expression was quantified by western blot analysis using whole liver homogenates from virgin and pregnant mice treated with vehicle or TCPOBOP. Representative western blots are shown and semi-quantification presented below. Data were normalized to virgin vehicle-treated controls (set to 1.0) and presented as mean relative expression ± SE (n=6-9). Black bars represent vehicle-treated mice, and light grey bars represent TCPOBOP-treated mice. Asterisks (*) represent differences ($p \leq 0.05$) compared with vehicle-treated virgin mice. Daggers (†) represent differences ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

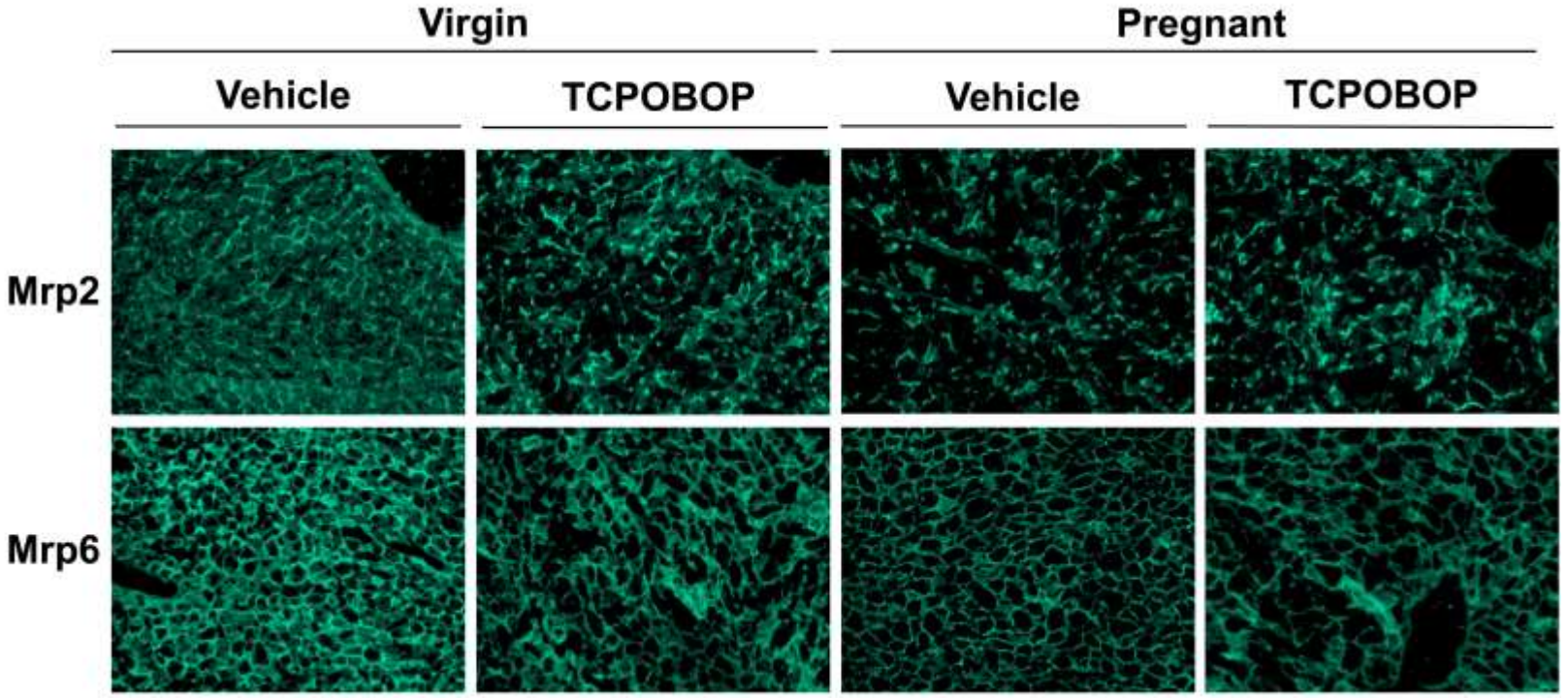


Fig. A-3.7. Indirect immunofluorescent staining of Mrp2 and Mrp6 in the livers of pregnant mice treated with TCPOBOP. Indirect immunofluorescence against canalicular transporter Mrp2 (*green*) and basolateral transporter Mrp6 (*green*) were conducted on liver cryosections (6 µm) obtained from virgin and pregnant mice treated with vehicle or TCPOBOP on gestation day 17. Representative images are shown. Magnification 200x.

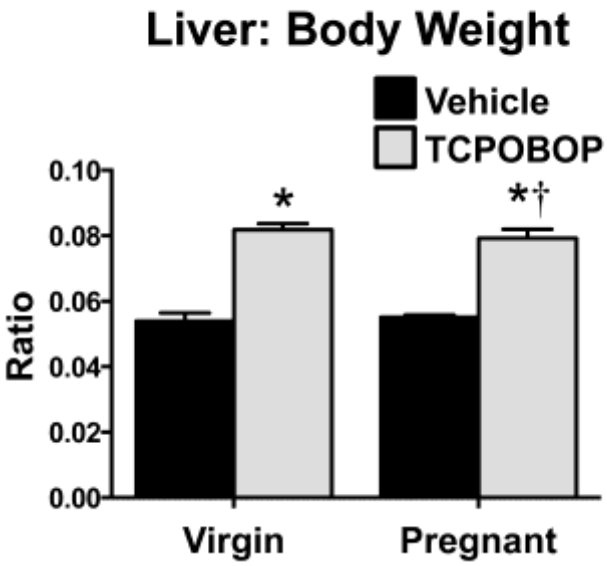


Fig. A-3.8. Liver weights in pregnant mice treated with TCPOBOP. Liver weights were expressed as a ratio to body weight in virgin and pregnant mice following treatment with TCPOBOP. Data were presented as mean ratios ± SE (n=6-9). Black bars represent vehicle-treated mice, and light grey bars represent TCPOBOP-treated mice. Asterisks (*) represent differences ($p \leq 0.05$) compared with vehicle-treated virgin mice. Daggers (†) represent differences ($p \leq 0.05$) compared with vehicle-treated pregnant mice.

Table A-3.1. qPCR primer sequences

Primer	Forward (5’ to 3’)	Reverse (5’ to 3’)
Car	ATCTGCCGCTCTTCCGGTCC	GGAACCCTGCATGGACTGCGT
Cyp2b10	TGCTGTCGTTGAGCCAACCTTCA	GGGGCTCCCTGGGATTTCCG
Ugt1a1	TGTCTTTCAACTCAGACCGC	AGATGCAGGGCTCAGAAGAT
Ugt1a6	AATTCAGATGCTGGCTGATG	AAGTGTCTGAGCAGCAGGAA
Ugt1a9	ACTGCCTCCAGAAGAAGTCA	CACAGGACCGTCTGAGGAA
Ugt2b5	GGCCAACCACCTTAGTTGAG	TTCCATATCCTTAGGCAAGGGTT
Gsta1	CCCCTTTCCCTCTGCTGAAG	TGCAGCTTCACTGAATCTTGAAAGC
Gsta2	CACACTCCTCTGGAGCTGGAT	CCCGGGCATTGAAGTAGTGA
Sult1a1	CCCGTCTATGCCCGGATAC	GGGCTGGTGTCTCTTTCAGAGT
Oatp1a1	CCAACGCAAGATCCAACAGAGTGTG	TCGGGCCAACAATCTTCCCCAT
Oatp1a4	CGTGGGGATACCGAATTGTCT	GCTTTTCCAAGATCAAGGCATTT
Mrp2	AGCAGGTGTTCGTTGTGTGT	AGCCAAGTGCATAGGTAGAGAAT
Mrp3	CTGGGTCCCCTGCATCTAC	GCCGTCTTGAGCCTGGATAAC
Mrp4	ACCCTCGTTGAAAGAC	TGAAGCCGATTCTCCCTTC
Mrp6	TGTCTGCAAGCCATCGGACTGTTTG	TGGAAAAGCGGTTCAGCAGGTTCC
Rpl13a	CAAGAAAAAGCGGATGGTGG	TCCGTAACCTCAAGATCT

APPENDIX 4: INDUCTION OF XENOBIOTIC METABOLIZING ENZYMES AND TRANSPORTERS IN HUMAN INTESTINAL CELLS BY PREGNANT SERUM

Jamie E Moscovitz[a], Guadalupe Herrera-Garcia[b], Grace L Guo[a], Lauren M Aleksunes[a]

[a] Department of Pharmacology and Toxicology, Rutgers University Ernest Mario School of Pharmacy, Piscataway, NJ

[b] Department of Maternal Fetal Medicine, Robert Wood Johnson Medical School, New Brunswick, NJ

A-4.1 Abstract

The intestine expresses many critical xenobiotic metabolizing enzymes and transporters that regulate oral absorption and fecal excretion. Important nutrients and vitamins are absorbed at this interface, and as a result expression of these enzymes and transporters is tightly regulated. While it is known that gastrointestinal motility decreases during pregnancy, additional molecular changes in intestinal cells have yet to be investigated. To determine whether pregnancy alters intestinal disposition pathways, RNA and protein were collected from human colon adenocarcinoma LS174T cells cultured in media supplemented with 20% serum from either non-pregnant or pregnant women (weeks 31 to 39 of gestation). In intestinal cells treated with pregnant serum, the mRNA expression of the CYP3A4 enzyme, basolateral efflux transporters OSTα and MRP3, as well as the luminal efflux transporters MDR1, MRP2 and BCRP are induced 2- to 4-fold as compared to cells treated with non-pregnant serum. MRP3 protein exhibits a 2-fold up-regulation when treated with pregnant serum. Profiling of differentially expressed genes (induction of CYP3A4 and MDR1 along with down-regulation of the SHP transcription factor) suggests that serum from pregnant women is activating the Pregnane X Receptor (PXR). Further mechanistic studies reveal that progesterone and its metabolite epiallopregnanolone-sulfate may be responsible, in part, for expression changes observed in cells treated with pregnant serum. Because MDR1, MRP2 and BCRP are important to the luminal efflux of endocrine disrupting chemicals, such as mycotoxins, perfluorinated chemicals, and pesticides, activation of intestinal PXR by hormones in pregnant serum may be an adaptive response to decrease systemic absorption and in turn, fetal exposure.

Abbreviations:

ASBT, apical sodium-dependent bile acid transporter; BCRP, breast cancer resistance protein; CYP, cytochrome P450; FGF, fibroblast growth factor; FXR, farnesoid x receptor; I-BABP, intestinal bile acid binding protein; MDR, multidrug resistance transporter; MRP, multidrug resistance-associated transporter; OST, organic solute transporter; PXR, pregnane x receptor; RXR, retinoid x receptor; SHP, small heterodimer partner

A-4.2 Introduction

Pregnancy changes the absorption and clearance of chemicals leading to altered pharmacokinetics for drugs and endogenous molecules. Greater than 50% of pregnant women take at least one medication, though the average is 3-5 prescriptions (Andrade et al., 2004; Jeong, 2010). Physiologically, there is decreased protein binding, enhanced hepatic blood flow and variable changes in hepatic enzyme activity during pregnancy (Hytten and Paintin, 1963; Pirani et al., 1973; Nakai et al., 2002; Creasy and Resnik, 2009). This is coupled with decreased gastrointestinal motility (Parry et al., 1970) that enables enhanced absorption of nutrients for placental and fetal growth, but may also alter xenobiotic disposition.

The Farnesoid X Receptor (FXR) and Pregnane X Receptor (PXR) are bile acid- and xenobiotic-activated transcription factors that belong to the nuclear receptor superfamily. FXR and PXR are highly expressed in the intestines, where they co-regulate downstream transcriptional regulators, xenobiotic metabolizing enzymes and uptake and efflux transporters, similar to their function in the liver. While the fibroblast growth factor (FGF) 19 has typically been associated with FXR regulation, it has been shown in LS174T human colon adenocarcinoma cells that activation of PXR induces FGF19 promoter activity and subsequent transcriptional up-regulation (Wistuba et al., 2007; Wang et al., 2011). Studies conducted in primary human hepatocytes revealed PXR-mediated activation of Cytochrome P450 (CYP) 3A4 occurs through suppression of small heterodimer partner (SHP), an important downstream target activated by FXR (Li and Chiang, 2006).

Treatment of human precision-cut ileal slices with lithocholic acid, both a ligand of FXR and PXR, induced apically located multidrug resistance-associated protein (MRP) 2 and

basolateral MRP3 efflux transporter expression (Khan et al., 2011). Interestingly, in Caco-2 cells, rifampicin but not the bile acid chenodeoxycholic acid up-regulated multidrug resistance transporter MDR1 and MRP2 (Martin et al., 2008). Studies in the LS174T as well as LS180 intestinal cell lines confirmed that PXR activation up-regulates MDR1 expression (Schuetz et al., 1996; Synold et al., 2001; Kota et al., 2010; Banerjee and Chen, 2014). While the same mechanism has not been confirmed in the intestines specifically, it has been demonstrated in primary human hepatocytes that breast cancer resistance protein (BCRP) is regulated by rifampicin activation of PXR (Jigorel et al., 2006).

Though little is known about the molecular adaptations in the intestine during pregnancy, steroid hormones released in large quantities in the third trimester have been implicated in hepatic changes to the Fxr pathway, and contribute to pregnancy-specific liver/cholestatic disease (Abu-Hayyeh et al., 2013a). Activation of estrogen receptor alpha (Erα) by estradiol leads to protein-protein complexes of Erα and Fxr and limits transcriptional activation of Fxr target genes, such as bile salt export pump (Song et al., 2014). Additionally, it has been reported that progesterone metabolites are partial agonists of Fxr, and prevent binding by full agonists such as bile acids during pregnancy, resulting in a global down-regulation of Fxr transcriptional target genes (Abu-Hayyeh et al., 2013a). Progesterone metabolites are also competitive substrates for the bile acid uptake transporter Na^+/taurocholate co-transporting polypeptide (Abu-Hayyeh et al., 2010). Finally, estradiol derivatives have been implicated in the post-translational modification of the hepatic Mrp2 transporter localization to the canalicular membrane, decreasing bile acid efflux into bile canaliculi (Mottino et al., 2005).

Immortalized colon carcinoma cell lines are commonly utilized in drug safety studies, as well as in the study of drug-drug interactions for transporters. This study sought to understand the interaction of the mixture of hormones present in the serum of pregnant women versus those of non-pregnant controls, in addition to employing a candidate based approach to comprehend the role of individual steroid hormones in the regulation of important transcriptional regulators of drug disposition, including xenobiotic and endobiotic transporters.

A-4.3 Materials and methods

Cell culture and treatment

Human colon adenocarcinoma LS174T cells (ATCC, Manassas, VA) were cultured in 12-well or 6-well plates with phenol red-free Minimum Essential Media (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum at 37ºC in an atmosphere containing 5% CO2. On day 4, cells were 1) treated with phenol red-free Minimum Essential Media containing 20% pooled non-pregnant or pregnant human serum (BioReclamation, Westbury, NY, Table A-4.1) for 48 hours or 2) cultured in media supplemented with 10% charcoal stripped fetal bovine serum and 100 μM chenodeoxycholic acid (Sigma, St. Louis, MO), and treated with vehicle, 1 μM progesterone (Sigma), 7 μM epiallopregnanolone-sulfate (PM5S, Steraloid, Newport, RI) or both progesterone and PM5S for 48 hours. For both types of experiments, media, total RNA and protein were collected on day 6.

Real-time quantitative PCR

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Complimentary DNA was made using High Capacity cDNA Synthesis for RT-PCR (Applied Biosystems, Foster City, CA). Quantitative analysis of mRNA was performed with SYBR Green fluorescence detection using a Viia 7 PCR system (Applied Biosystems). Ribosomal protein L13A (RPL13A) was used as a reference gene. Ct values were converted to delta delta Ct values by comparing to RPL13A. Specific forward and reverse primers are listed in table A-4.2.

Western blot analysis

Protein expression was determined from whole cell lysates by western blot analysis with chemiluminescent detection. Semi-quantification of expression was determined using

primary antibodies raised against MDR1 (1:2000, C-219, Abcam, Cambridge, MA) and MRP_3II-9 (1:2000, Enzo Life Sciences, Farmingdale, NY). Beta-actin (1:2000, Abcam) was used as a loading control. Representative images are shown. Densitometry was performed using AlphaView SA (ProteinSimple, San Jose, CA).

FGF19 protein quantification

FGF19 protein secretion was quantified in cell culture medium using a human ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis

Quantitative results were expressed as mean ± standard deviation (SD). Data were analyzed using a two-tailed, unpaired student's t-test or a 1-way ANOVA followed by a Newman-Keuls multiple comparison post-hoc test using GraphPad Prism (V6) (GraphPad, La Jolla, CA). Significance was set at $p \leq 0.05$.

A-4.4 Results

Nuclear receptor mRNA expression in intestinal cells treated with pregnant serum.

Neither mRNA expression of PXR or FXRα was altered in cells treated with pregnant serum (Fig. A-4.1). However, both the heterodimerizing partner of each nuclear receptor, RXRα, and co-regulated transcription factor SHP were reduced 20 and 25% in the presence of pregnant serum, respectively. CYP3A4, the prototypical target enzyme of PXR was inducible by 3.3-fold in pregnant serum-treated cells when compared to non-pregnant serum-treated controls.

Regulation of intestinal reabsorption in cells treated with pregnant serum.

In the intestine, FGF19 is co-regulated by both PXR and FXR (Wistuba et al., 2007; Wang et al., 2011). In cells treated with pregnant serum, FGF19 mRNA levels were induced 4.6-fold (Fig. A-4.2). Similar to mRNA, FGF19 protein secretion was enhanced 50% with pregnant-plasma treatment. Of the target transporters transcriptionally regulated by FXR, OSTα mRNAs increased by 2.5-fold (Fig. A-4.3). Notably, the mRNA expression of all PXR-regulated transporters, including MDR1, MRP2, MRP3 and BCRP was enhanced 2- to 4-fold in pregnant serum treated intestinal cells compared to controls. Induction of MRP3 protein (2.8-fold) was also observed, as determined by western blot and semi-quantification (Fig. A-4.4).

Regulation of nuclear receptor pathways in intestinal cells treated with progestins.

Expression of FXRα was reduced by 25% with co-treatment of progesterone and the progesterone metabolite PM5S (Fig. A-4.5). Treatment with individual or a combination of progestins did not alter FXR or PXR prototypical target genes, or the nuclear receptors RXRα and SHP. Co-treatment with the combination of progesterone and PM5S induced both FGF19 mRNA expression (2.5-fold) and protein secretion into media

(2-fold) (Fig. A-4.6). The gene expression of MRP2 and MRP3 were also induced by combination treatment of progesterone and PM5S, 1.5- and 2-fold, respectively (Fig. A-4.7).

A-4.5 Discussion

Studies conducted in human colon adenocarcinoma LS174T cells demonstrate that supplementation of media with 20% human pregnant serum robustly induces gene expression of PXR-regulated enzymes and transporters, including CYP3A4, MDR1, MRP2, BCRP and MRP3, as compared to non-pregnant serum-treated controls. Alternatively, the nuclear receptors RXRα and SHP are repressed. FXR target genes appear to be variably affected by treatment with pregnant serum in comparison to non-pregnant serum. Gene expression and protein secretion of the co-regulated endocrine factor FGF19 were induced in pregnant serum-treated versus non-pregnant serum-treated cells. Further, progestins that reach high levels in the third trimester of pregnancy were investigated in a candidate hormone approach, and indicated that the induction of FGF19, MRP2 and MRP3 may be related to progestin-mediated activation of PXR.

LS174T cells treated with candidate steroid hormones were primed with the bile acid chenodeoxycholic acid to control for the differences between treatments with human serum and supplementation of bovine serum. Previous literature suggests that levels of chenodeoxycholic acid are comparable between non-pregnant and pregnant women (Castano et al., 2006; Egan et al., 2012). The co-treatment of progesterone and its metabolite, PM5S, were able to significantly alter expression of PXR target transporters MRP2 and MRP3 in LS174T cells (Fig. A-4.7). It has been observed that endocrine and orphan nuclear receptor activation by steroid hormones is dose dependent (reviewed in Jeong, 2010). Therefore, for this candidate hormone approach, concentrations utilized for these experiments, 1 μM for progesterone, and 7 μM for PM5S, are reflective of those observed in the third trimester of human pregnancy (Abu-Hayyeh et al., 2010; Jeong, 2010). While the authors anticipate that the induction of xenobiotic transporters by progestins is through PXR (Blumberg et al., 1998; Kliewer et al., 1998), additional

studies conducted in LS174T cells identify a mechanism of BCRP activation that is FXR-dependent (Herraez et al., 2012). Treatment of LS174T cells transfected with FXR with 1 μM GW4064, a potent and specific FXR agonist, for 24 hours resulted in induction of BCRP and not MRP2. However, no induction of BCRP and MRP2 gene expression was observed with GW4064 treatment in the absence of transfection (Herraez et al., 2012). As PXR is more highly expressed in colon than FXR, it is possible that FXR activation is masked in the model system used for these studies.

Immortalized colon cells, in particular Caco-2 cells, are commonly employed to investigate drug drug interactions. The current study relied upon LS174T cells, which have been gaining popularity as a colon cell line that more stably and consistently expresses nuclear receptors and transporters as compared to the Caco-2 cell line (Anderle et al., 1998; Goto et al., 2003; Pfrunder et al., 2003). Though LS174T cells are comparatively better than Caco-2 cells to study nuclear receptor signaling pathways, they are still representative of the colon. Therefore, these data may only be applicable to drugs absorbed in the large intestine, which is most often secondary to small intestine absorption. There are currently few *in vitro* models of small intestine, including primary epithelial cells, which have not been characterized for use to study xenobiotic transporters.

Interestingly, consistent among replicates and independent of passage number, OSTβ mRNA was detectable in experiments conducted with non-pregnant and pregnant human serum, but not detectable in studies conducted with fetal bovine serum supplementation. A recent study utilized time dependent inhibition assays to test the accurate prediction of protein kinase inhibitors and CYP3A in plated cryopreserved human hepatocytes, human hepatocytes cultured in Dulbecco's Modified Eagle Medium,

and human hepatocytes suspended in human plasma (Mao et al., 2016). Human hepatocytes suspended in human plasma exhibited the lowest CYP3A inactivation potency (as determined by the inactivation constant/apparent inactivation constant). Further, they were able to most accurately predict (95%) the clinically observed drug drug interactions for 10 of the 19 protein kinase inhibitors with CYP3A (Mao et al., 2016). More work should be conducted on the clinical relevance and reliability of *in vitro* systems with human serum supplementation as opposed to other species.

Deciphering the individual contributions of drug metabolizing enzymes and transporters in the liver and intestine to drug disposition changes observed during pregnancy is a challenging pursuit. Accessibility to the pregnant population, as well as the invasiveness of pharmacokinetic profiling and ethical concerns limit the availability of this information. *In vitro* studies presented in this report suggest that activation of intestinal PXR may contribute to altered pharmacokinetic profiles of drugs used during pregnancy. Taken together, these data support the investigation of the milieu of hormones late in gestation that may result in alterations to intestinal drug metabolizing enzymes and transporters. Further, these findings suggests greater attention should be given to enhanced expression of apical efflux transporters as a mechanism of reduced bioavailability of orally administered drugs in pregnancy. Future studies should focus on conducting ABC-transporter functional assays in the presence of pregnant and non-pregnant serum to determine if altered expression leads to altered function.

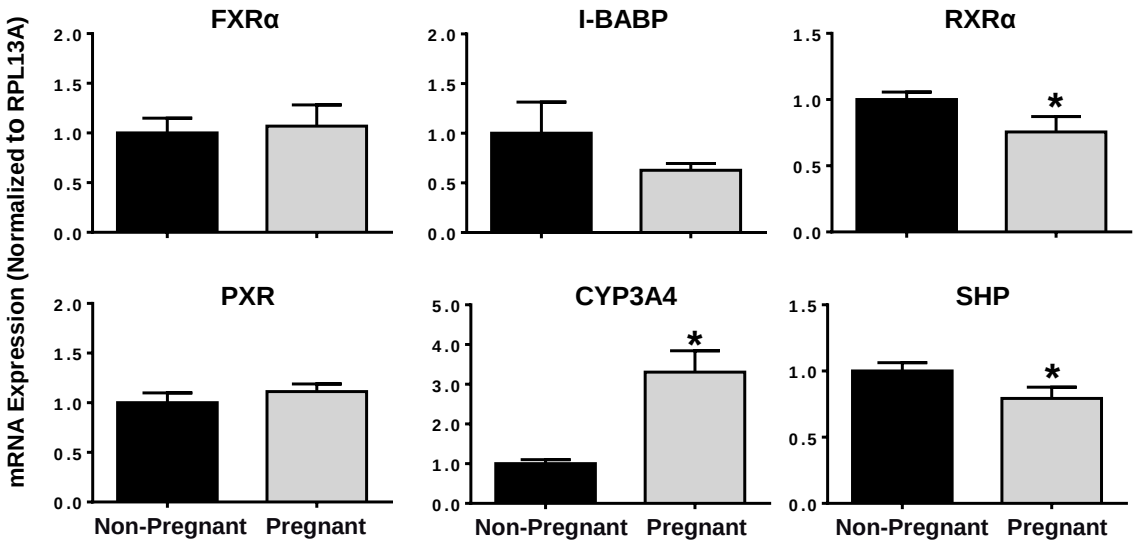


Fig. A-4.1. Nuclear receptor mRNA expression in intestinal cells treated with pregnant serum. mRNA expression of intestinal nuclear receptors and prototypical target genes was quantified in LS174T cells treated with 20% non-pregnant or pregnant human serum for 48 hours. Bar = mean relative expression ± SD (n=3 experiments/3 replicates). *p≤0.05 compared to control.

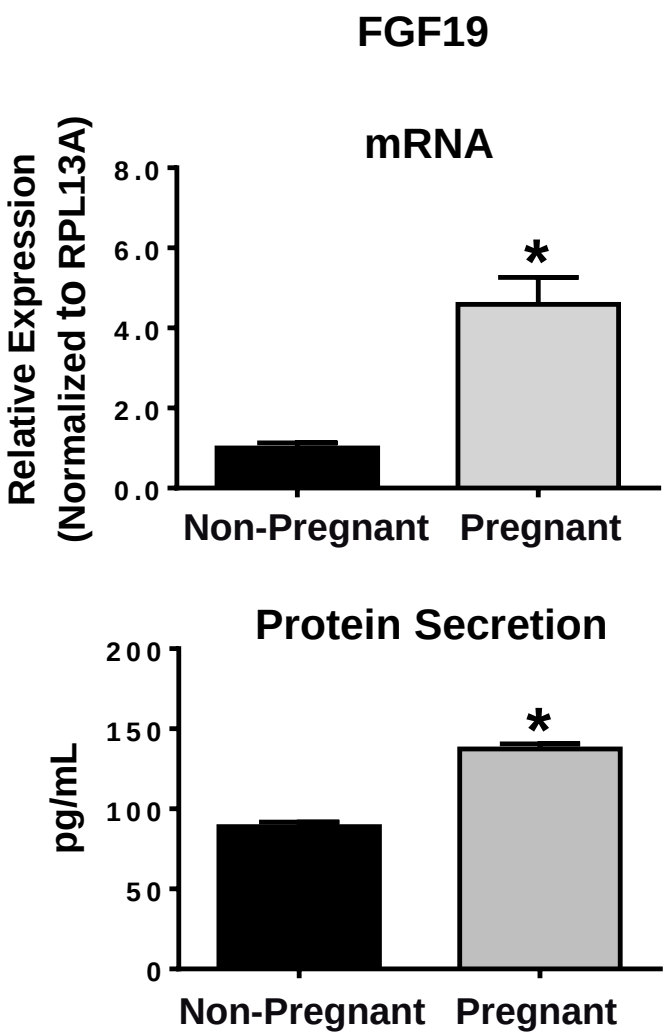


Fig. A-4.2. Regulation of intestinal FGF19 by pregnant serum. mRNA and protein expression of endocrine factor FGF19 in LS174T cells treated with 20% non-pregnant or pregnant human serum for 48 hours. FGF19 protein secretion was measured using an ELISA kit. Bar = mean relative expression ± SD (n=3 experiments/3 replicates). *p≤0.05 compared to control.

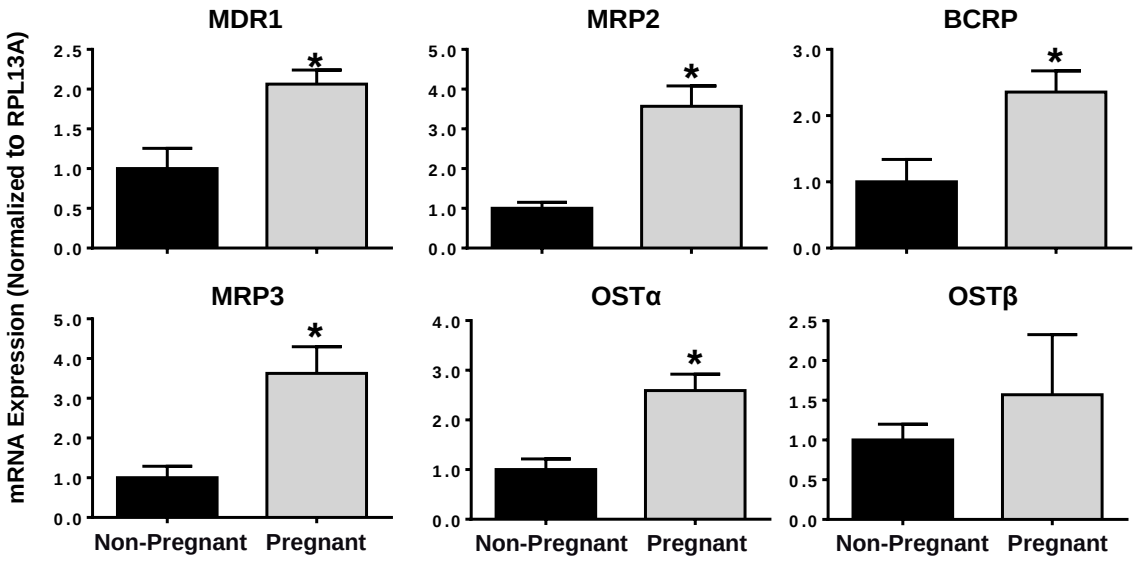


Fig. A-4.3. Regulation of intestinal efflux transporter genes by pregnant serum. mRNA expression of apical and basolateral efflux transporters in LS174T cells treated with 20% non-pregnant or pregnant human serum for 48 hours. Bar = mean relative expression ± SD (n=3 experiments/3 replicates). *p≤0.05 compared to control.

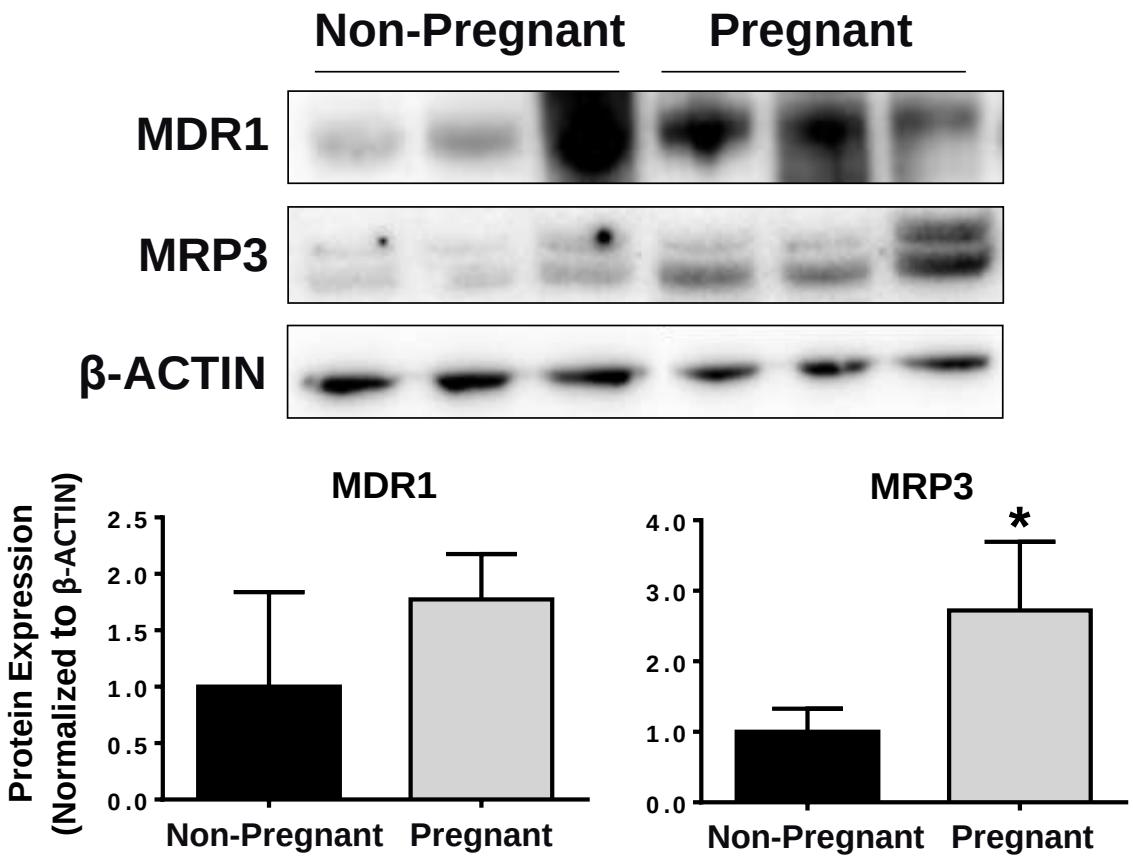


Fig. A-4.4. Regulation of intestinal efflux transporter protein by pregnant serum. **P**rotein expression of efflux transoprteres in LS174T cells treated with 20% non-pregnant or pregnant human serum for 48 hours. Western blot band intensities were semi-quantified. Representative images are shown. Bar = mean relative expression ± SD (n=3 experiments/3 replicates). *p≤0.05 compared to control.

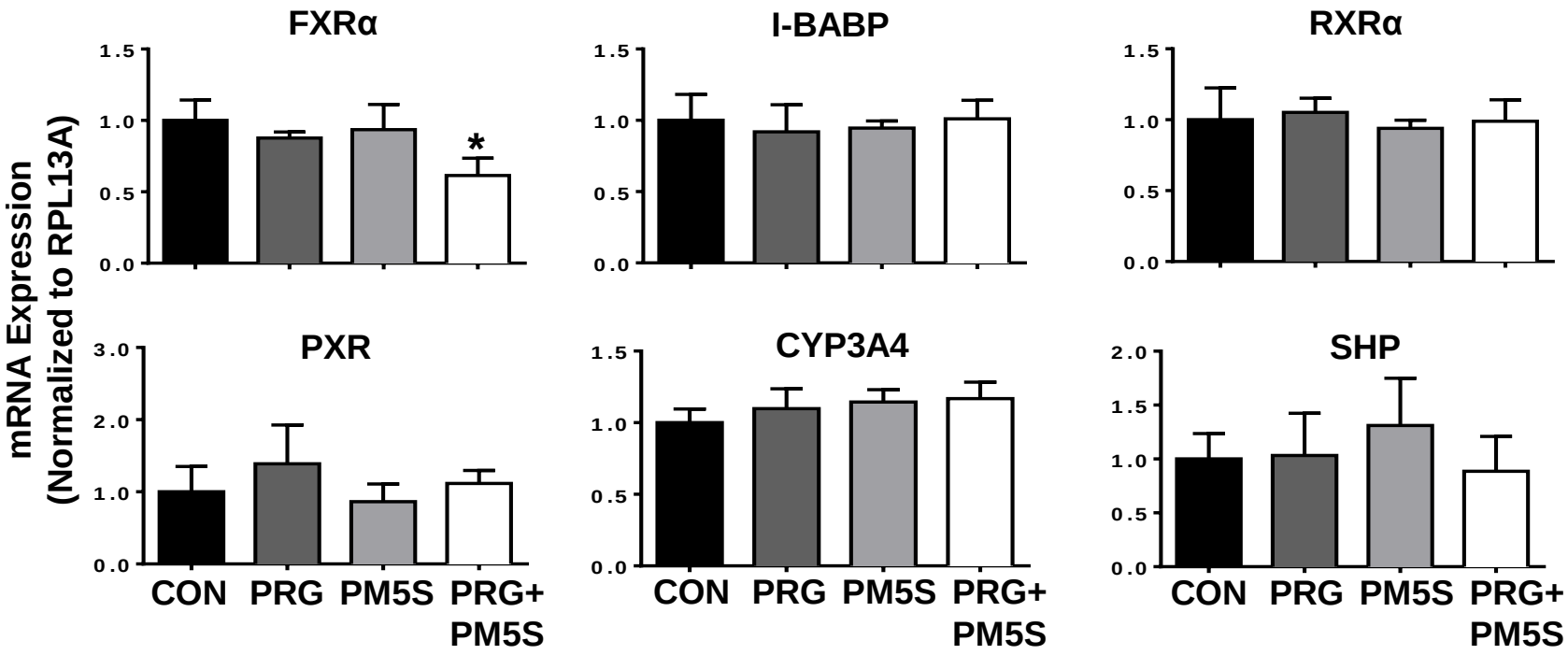

FXRα
I-BABP
RXRα
PXR
CYP3A4
SHP
mRNA Expression
(Normalized to RPL13A)
*
CON
PRG
PM5S
PRG+
PM5S

Fig. A-4.5. Regulation of intestinal reabsorption genes by progestins. mRNA expression of intestinal FXR and PXR targets was quantified in LS174T cells treated with vehicle, progesterone (PRG, 1 μM), epiallopregnanolone sulfate (PM5S, 7 μM) or PRG and PM5S for 48 hours. Bar = mean relative expression ± SD (n=3 experiments/3 replicates). *p≤0.05 compared to control.

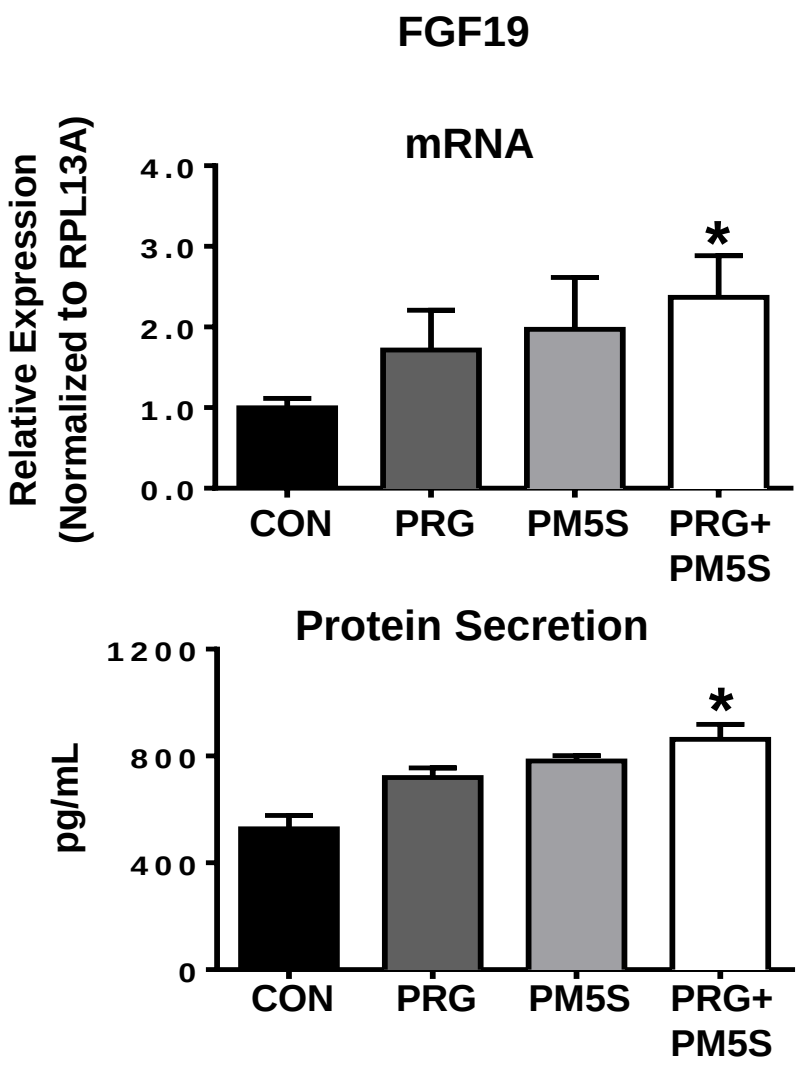


Fig. A-4.6. Regulation of Intestinal FGF19 by progestins. Expression of intestinal endocrine factor FGF19 was quantified in LS174T cells treated with vehicle, progesterone (PRG, 1 μM), epiallopregnanolone sulfate (PM5S, 7 μM) or PRG and PM5S for 48 hours. Bar = mean relative expression ± SD (n=3). *p≤0.05 compared to control.

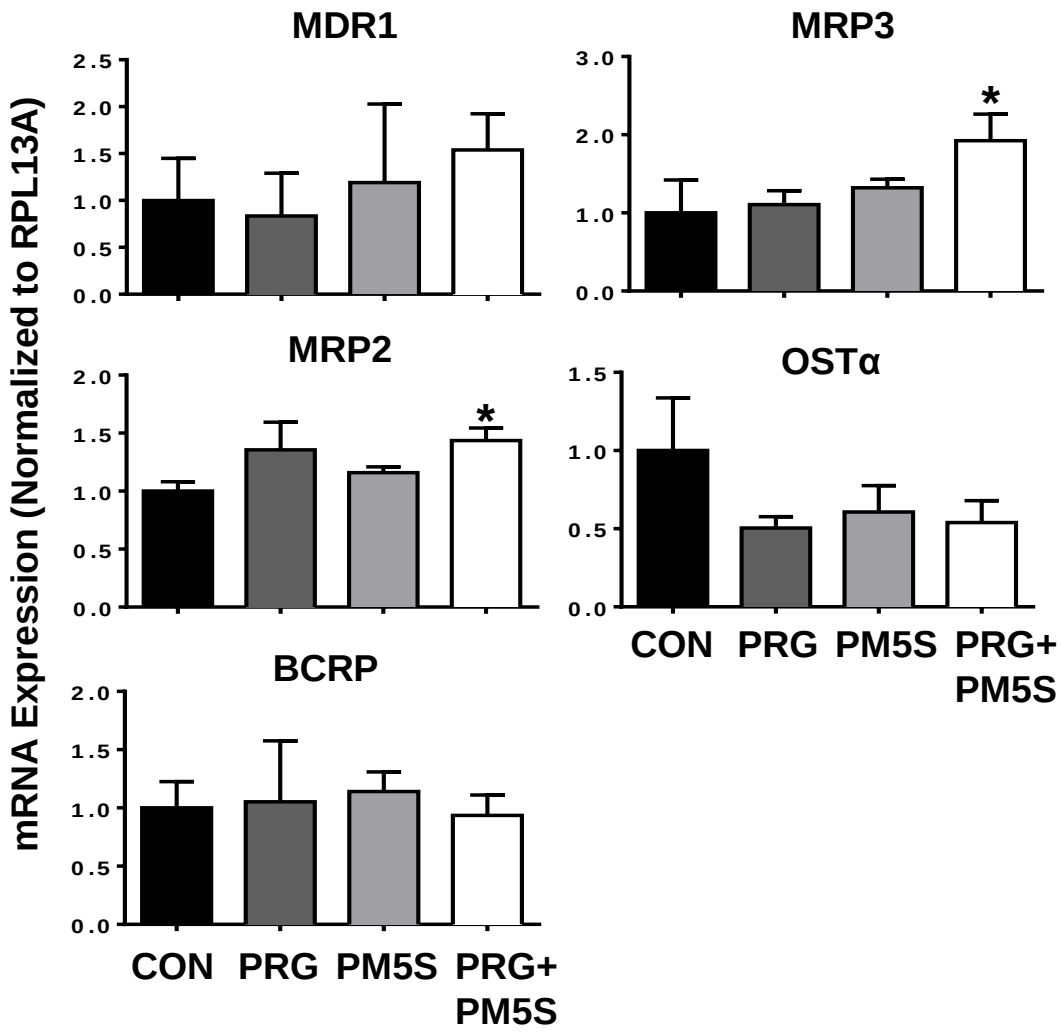


Fig. A-4.7. Regulation of intestinal transporter genes by progestins. Expression of intestinal apical and basolateral efflux transporter genes was quantified in LS174T cells treated with vehicle, progesterone (PRG, 1 μM), epiallopregnanolone sulfate (PM5S, 7 μM) or PRG and PM5S for 48 hours. Bar = mean relative expression ± SD (n=3). *p≤0.05 compared to control.

Table A-4.1. Serum donor information[1]

	Non-Pregnant	Pregnant	
Donor	**Age**	**Maternal Age**	**Gestation (weeks)**
1	24	24	32
2	28	24	36
3	30	26	36
4	30	30	39
5	31	32	31
6	--	32	34

[1]Available information on serum donors for pooled serum treatments

Table A-4.2. qPCR primer sequences

Primer	Forward (5’ to 3’)	Reverse (5’ to 3’)
CYP3A4	CACAAACCGGAGGCCTTTTGGTC	GTCTCTGCTTCCCGCCTCAGAT
FGF19	TCGGAGGAAGACTGTGCTTTC	CCTCTCGGATCGGTACACATTG
FXRα	CGCCTGACTGAATTACGGACA	TCACTGCACGTCCCAGATTTC
I-BABP	CTCCAGCGATGTAATCGAAA	CCCCCATTGTCTGTATGTTG
MDR1	TTGAAATGAAAATGTTGTCTGG	CAAAGAAACAACGGTTCGG
MRP2	AGCCATGCAGTTTTCTGAGGCCT	TGGTGCCCTTGATGGTGTGC
MRP3	CTTCCTGGTGACCCTGATCACCCT	TGCTGGATCCGTTTCAGAGACACA
OSTα	GGAGCACAGCTCTATGGATCA	TCAGGATGAGGAGAACCTGGA
PXR	ATCTCCTACTTCAGGGACTT	AGCTTCTTCAGCATGTAGTG
RPL13A	GGTGCAGGTCCTGGTGCTTGA	GGCCTCGGGAAGGGTTGGTG
RXRα	CTTGGGAACTTTGTCGTTTC	CCAACAGCACTGTACAACTA
SHP	ATCCTCTTCAACCCCGATGTG	GTCGGAATGGACTTGAGGGT

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