UNIVERSITY OF CALIFORNIA, SAN DIEGO

Development and Analysis of Nuclear Receptor Mouse Models in the Study of Drug Metabolism and Neonatal Jaundice

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The thesis of Dallin Schow Lindahl is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVATIONS AND SYMBOLS

3'-E	460 bp product near the 3'-end of the BAC clone
5'-E	334 bp product near the 5'-end of the BAC clone
ADR	adverse drug reactions
AhR	aryl hydrocarbon receptor
BAC	bacterial artificial chromosome
bp	base pair
CAR	constitutive androstane receptor
CN-I	Crigler-Najjar type I
CN-II	Crigler-Najjar type II
CPH	cyclophilin
Ct	cycle threshold
DBD	DNA-binding domain
DME	drug metabolizing enzymes
Ex1	208 bp PCR product ~700 bp downstream of exon 1
Ex5	164 bp product within exon 5
Ex9	214 bp PCR product ~200 bp downstream of exon 9
GS	Gilbert's syndrome
hPXR	human PXR
*hUGT1*1*	humanized *UGT1*1*
kb	kilobase pair

LBD	ligand-binding domain
LXR	liver X receptor
NcoR	nuclear receptor co-repressor
NRs	nuclear receptors
P450	cytochrome P450 enzymes
PCR	polymerase chain reaction
PPARs	peroxisome proliferator activated receptors
PR	591 bp product ~6500 bp upstream of exon 1
PXR	pregnane X receptor
qPCR	quantitative real-time PCR
RIF	rifampicin
RXR	retinoid X receptor
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
SNP	single-nucleotide polymorphism
UDPGA	UDP-glucuronic acid
UGTs	UDP-glucuronosyltransferases
XR	xenobiotic receptors

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ABSTRACT OF THE THESIS

Development and Analysis of Nuclear Receptor Mouse Models in the Study of Drug Metabolism and Neonatal Jaundice

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Understanding the relationship between nuclear receptors and drug metabolizing enzymes (DMEs) is important because it is closely associated with the metabolism of endogenous and xenobiotic compounds as well as drug-drug interactions. This study is divided into two parts: 1) the creation of a PXR transgenic mouse model and 2) the regulation of the *UDP*-

glucuronosyltransferase 1A (UGT1A) locus through activation of the liver x receptor α (LXRα). The transgenic mouse line that contains the human pregnane X receptor (hPXR) transgene was established and the PXR transgene was determined to be functional by elevated levels of cytochrome p450 3a11 (Cyp3a11) in response to hPXR activator rifampicin. Crossing this transgenic mouse line with the mouse line *Tg(UGT1^{A1*1})Ugt$^{-/-}$Pxr$^{-/-}$ (hUGT1*1/Pxr$^{-/-}$)* line, previously established in the lab, we created a *Tg(UGT1^{A1*1})Ugt$^{-/-}$Tg(Pxr)Pxr$^{-/-}$ (hUGT1*1/hPXR)* mouse line. For the second aim of this study, humanized UGT1A (hUGT1) mice were administered the LXR agonist, T090137, and the results show a significant induction of UGT1A1 in the liver and intestinal tissues compared with the control mice, suggesting that the LXRα regulates the *UGT1A* locus. These results are further supported by a significant reduction of serum bilirubin levels in response to T090137 treatment. Work is ongoing to create *Lxrα-null* mice on a *hUGT1A* background (hUGT1/$^{-}$ Lxr$^{-/}$ mice). In conclusion, these two mice lines, *hUGT1*1/hPXR* and *hUGT1A/Lxrα$^{-/-}$*, are potentially useful to study the roles of PXR and LXR in the *UGT1A* locus regulation that may have significant implications in altering glucuronidation activity of xenobiotics and endogenous compounds, including bilirubin.

CHAPTER 1:

Introduction

INTRODUCTION

Drug Metabolizing Enzymes

Drug metabolizing enzymes (DME) are responsible for the metabolism and elimination of many endogenous and exogenous compounds, known as xenobiotics. Metabolism is carried out through the collaboration of phase I and phase II enzymes. Phase I enzymes consist of multiple families of enzymes that typically act through simple oxidation, reduction, or hydrolysis reactions in order to introduce a reactive and polar functional group to their substrates. The polar functional groups can help facilitate the direct elimination of substrates or make them more suitable for conjugation by phase II enzymes, ultimately leading to elimination through urine or feces (Poulos, 2005). The majority of phase I reactions are carried out by the cytochrome P450 (P450) family of enzymes. P450s are a superfamily of heme-dependent monooxygenases and consist of many isoforms across different species (Poulos, 2005). P450s function through an oxidation reaction that uses NADPH as co-factor to place a polar oxygen species on lipophilic or aliphatic compounds (Poulos, 1998). P450s are highly expressed in the liver and intestine and are largely responsible for the first pass metabolism of many pharmaceutical drugs (Poulos, 2005; Lehmann *et al.,* 1998). The most important human P450 that is associated with drug metabolism is CYP3A4, which is responsible for the metabolism of ~50% of pharmaceutical agents (Guengerich, 1999).

UDP-glucuronosyltransferases

UDP-glucuronosyltransferases (UGTs) belong to the class of phase II enzymes that are responsible for the conjugation of various endogenous and exogenous compounds. Phase II metabolism is defined as the conjugation of large, water soluble groups to reactive substrates to facilitate their excretion through bile or urine (Guillemette, 2003). UGTs utilize UDP-glucuronic acid (UDPGA) as a co-substrate and facilitate the addition of glucuronic acid to a nucleophile substrate, thus creating a glucuronide with increased polarity (Guillemette, 2003). The glucuronides are more readily excreted through the bile or urine due to the increased water solubility and can be recognized by the renal transport system (Dutton, 1980). UGTs are localized within the lumen side of the endoplasmic reticulum membrane and play an important role in the metabolism of almost all classes of drugs, accounting for the metabolism of approximately one-third of all drugs metabolized by phase II enzymes. UGTs are also known to provide protection against toxic dietary and environmental chemicals and play a key role in the homeostasis of endogenous compounds that include hormones, bilirubin and fatty acids (Belanger *et al.,* 1998; Jude *et al.,* 2001). In most cases, the addition of the UDPGA leads to the resulting glucuronide being biologically inactive. With some exceptional compounds, such as morphine, the respective glucuronides lead to an increase in their biological activity.

The UGT superfamily consists of eighteen functional proteins, divided into two families, the *UGT1* and *UGT*2, based on the homology of their DNA sequences (Burchell *et al.,* 1991). The UGT1 proteins are largely responsible for the metabolism of various xenobiotics and some endogenous substrates that include bilirubin. Contrasted to the UGT1 family, UGT2 proteins are largely responsible for the metabolism of endogenous substrates that include steroids and bile acids, and they are also involved in the metabolism of some xenobiotics. The *UGT1* locus, located on chromosome 2 (2q37), possesses a gene locus organization that is unique in biology, is composed of 17 exons, and spans approximately 210 kb (Owens *et al.*, 1995; Ritter *et al.,* 1995; Gong *et al.,* 2001). The *UGT1A* locus encodes 13 proteins, with 9 that are functional (UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10) and 4 that are pseudogenes (UGT1A2P, UGT1A11P, UGT1A12P, and UGT1A13P). Each UGT1 gene contains five exons encoding 527-530 amino residues (Owens *et al.*, 1995; Ritter *et al.,* 1995; Gong *et al.,* 2001). Transcription of the *UGT1* locus proceeds through a process known as exon sharing: an individually unique exon 1 for each isoform is spliced and joined to exons 2-5, a common region that is identical to all UGT1 isoforms. The exon 1 sequence of each isoform is flanked by its own functional promoter that independently regulates transcription of the respective protein (Tukey and Strassburg, 2000). The exon 1 sequence determines substrate specificity of the UGT1 proteins, while the common 2-5 exons

encode the region containing the co-substrate UDPGA binding domain (Guillemette, 2003). The human *UGT2* family consists of nine genes, and, in contrast to the *UGT1* family, each UGT2 gene encodes a single gene product. The *UGT2* genes are split into two sub families, *UGT2A and UGT2B,* and encode UGT proteins including UGT2B7, UGT2B11, UGT2B28, UGT2B10, UGT2B15, UGT2B17, UGT2B4, UGT2A1, and UGT2A2 (Guillemette, 2003). Each UGT2 gene contains 6 exons, with exons 3-6 being highly homogenous, whereas exons 1 and 2, encoding the N-terminal portion of the protein, are more divergent in DNA sequences. In UGT2 genes, similar to the UGT1s, the carboxyl region contains the UDPGA binding domain, and the N-terminal region determines substrate specificity of each protein (Monaghan *et al*, 1994).

The liver is known to be the major site of glucuronidation; however, extrahepatic glucuronidation in other organs may also play a key role in the overall metabolism, depending on the substrates (Chen et al., 2013). With UGTs playing an important role in the defense system of humans and other organisms, UGTs are expressed at the major sites of entry for foreign substances, including the skin, lung, epithelial lining of the nasal cavity, gut, and white blood cells (Tukey and Strassburg, 2001). They are also expressed in a variety of tissues and organs including the brain, kidney, heart, and prostate (Tukey and Strassburg, 2001). The predominant UGT1 isoforms expressed in the liver are UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9 (Strassburg *et al*., 1997a). UGT1A8 and UGT1A10, absent in the

liver, are both detected in the small intestine (along with UGT1A1, UGT1A4, UGT1A5, and UGT1A6) and colon. The colon has the most diverse expression pattern, expressing all UGT1 genes except UGT1A5 and UGT1A7 (Tukey and Strassburg, 2001). The expression of UGT1A7 is limited to the upper GI tract including the esophagus and stomach (Strassburg *et al*., 1997b).

The UGT2 genes also show a tissue-specific regulation pattern similar to the UGT1s but differentiate in the lack of definite distinction between hepatic and extrahepatic expression. UGT2A1 expression is limited to the sensory tissue and is not found in the liver or GI tract (Jedlitschky *et al*., 1999). UGT2B7, UGT2B10, UGT2B15, and UGT2B17 are expressed in the liver, GI tract, and steroid target tissues (Beaulieu *et al.*, 1996). UGT2B4 is expressed in the liver and polymorphically expressed in the small intestine (Strassburg *et al.,* 2000). The glucuronidation of some substrates, for example paracetamol, can be carried out by multiple DMEs. In contrast, there are substrates, such as bilirubin, that are metabolized by a single DME. The tissue-specific regulation of *UGT* genes and their substrate specificity gives further evidence that tissue-specific expression of each UGT isoenzyme plays a key role in the overall glucuronidation abilities of the human body.

DME Polymorphisms, Disease, and Adverse Drug Reactions

Advances in the field of pharmacogenomics have established a correlation between genetic factors and the expression of DMEs. Common genetic alterations, known as polymorphisms, are the result of small insertions

or deletions, or single-nucleotide polymorphisms (SNPs) (Guillemette, 2003). Polymorphisms can be observed throughout the gene, including the regulatory and coding sequences. Polymorphisms of the DMEs can alter the structure, function, or expression of the proteins, leading to changes in phenotype, ranging from barely noticeable to lethal. Due to the diverse biological functions of UGTs, their polymorphisms have the potential to alter biological pathways involving the metabolism of drugs, carcinogens, and endogenous substrates (Guillemette, 2003). The alteration in *UGT* genes resulting in varying pharmacokinetic profiles of their substrates that are clinical drugs is associated with adverse drug reactions (ADR). The role genetics plays in the glucuronidation of hormones, carcinogens, and dietary components is not well understood, but there is evidence that certain polymorphisms play a role in an individual's resistance or susceptibility to certain diseases (Guillemette *et al., 2000;* Tukey and Strassburg, 2000).

UGT1A1

UGT1A1 is an important *UGT1* gene product due to its role in the glucuronidation of bilirubin and xenobiotics. Bilirubin is a byproduct of heme metabolism and at high serum levels can cause damage to the central nervous system, with serum levels around 20 mg/dL being potentially lethal (Fujiwara *et al*., 2010). Due to bilirubin being conjugated solely by UGT1A1, polymorphisms within the *UGT1A1* gene locus can have dramatic effects. Although Crigler-Najjar type I (CN-I) and Crigler-Najjar type II (CN-II) are rare,

they are associated with severe *UGT1A1* gene mutations resulting in deficiency in bilirubin glucuronidation that manifests as severe hyperbilirubinemia. CN-1 is the more severe of the two types and results in serum bilirubin levels of 20-50 mg/dL (Crigler and Najjar, 1952). Due to the absence of the functional UGT1A1 enzyme, patients with CN-1do not have the ability to conjugate bilirubin. Phototherapy treatment under UV light can extend life expectancy of those affected by CN-1, with a liver transplant being the only permanent treatment (Gourley, 1997). CN-II is characterized by a decrease in UGT1A1 activity, leading to serum bilirubin levels of approximately 7-20 mg/dL. CN-II can be treated with phenobarbital, which leads to induction of the UGT1A1 enzyme. CN-I and CN-II can be caused by mutations to exons 1-5, with mutations on exons 2-5 affecting all genes transcribed from the *UGT1* locus.

Less severe mutations also exist. For example, Gilbert's syndrome (GS), caused by an atypical TATA-box region of the *UGT1A1* promoter, is a common polymorphism that results in mild hyperbilirubinemia. The wild-type (UGT1A1*1) promoter of the *UGT1A1* gene contains six repeating TA sequences in the TATA-box, compared to individuals with GS (UGT1A1*28) that have seven repeating TA sequences. Studies have demonstrated that increasing the number of TA sequences in the promoter region leads to a decrease in transcription of the *UGT1A1* gene, resulting in decreased glucuronidation activity (Guillemette *et al., 2000*; Beutler *et al., 1998;* Bosma *et*

al., 1994). GS is classified as being homozygous for the UGT1A1*28 mutant allele and is found in 6-12% of the population. Clinically, GS contributes to prolonged neonatal jaundice, slightly elevated bilirubin levels throughout life, and ADR (Bosma *et al.,* 1995). However, GS is also associated with a decreased risk of cardiovascular and liver disease (Bosma *et al.,* 1995).

UGT1A1 polymorphisms that lower glucuronidation activity, including the UGT1A1*28 variant found in patients with GS, have been linked to ADR and are associated with some forms of cancer. It has been reported that individuals with GS have a lowered glucuronidation rate of various therapeutic drugs, including lorazepam, acetaminophen, tolbutamide, and irinotecan. With lowered glucuronidation rates, the potential for ADR with some medications is increased, especially for therapeutic agents that have a small therapeutic index. It has been demonstrated that patients with the UGT1A1*28 allele are more susceptible to having a toxic reaction to some anticancer drugs, including irinotecan (Iyer *et al.,* 1999). Irinotecan, also known as CTP-11, is a pro-drug that is used as the initial treatment for metastatic colorectal cancer. CTP-11 is metabolized by carboxylesterases into the active metabolite, SN-38, which is responsible for the therapeutic and toxic effects of the drug. SN-38 is detoxified into an inactive metabolite, SN-38-glucornoide, primarily through glucuronidation by the UGT1A1 enzyme (Gupta *et al.,* 1994). Patients with GS are more likely to experience the toxic effects of SN-38, mainly diarrhea and neutropenia, due to lower UGT1A1 activity. Genetic variations in the *UGT1A1*

locus have also been linked to a greater susceptibility to steroid related cancers. Studies have revealed that women with low transcriptional activity alleles (UGT1A1*28 and UGT1A1*34) were positively linked to breast cancer (Guillemette *et al.,* 2000).

Receptors

The evolution of more complex organisms created a need for such organisms to regulate increasingly complex biological functions, including the elimination of endogenous and exogenous substrates. Receptors evolved in order to receive chemical signals from outside the cell and transmit the signal throughout the cell or tissue. When the appropriate substrate binds to a receptor, it creates a cellular response that affects a specific biological function. Receptors are broadly split into four classes: 1) ligand-gated ion receptors 2) G protein-coupled receptors 3) kinase receptors and 4) nuclear receptors (NRs). Receptors that are involved in the sensing of toxic metabolites, from either endogenous or exogenous sources, are termed xenobiotic receptors (XR). Examples of xenobiotic receptors include the liver X receptor (LXR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), peroxisome proliferator activated receptors (PPARs), and aryl hydrocarbon receptor (AhR) (Tolson and Wang, 2010). The xenobiotic receptors transform stimuli from endogenous and exogenous substrates into cellular responses through the regulation of their target genes. Thus, xenobiotic receptors play an essential role in protecting the body from toxic

metabolites through the regulation of DMEs (Li *et al*., 1998).

NRs are a super family of receptors that were originally discovered as orphan receptors and regulate expression of their target genes by acting as transcriptional factors that bind to respective consensus DNA response elements in response to ligand binding (Evans and Mangelsdorf, 2014). NRs all share a similar structure composition, containing a well-conserved C-terminal and a cysteine-rich central domain, with a variable N-terminal region (Evans and Hollenberg, 1988). The C-terminal half of the receptor has been demonstrated to be the ligand-binding domain (LBD) of the receptor and is responsible for the recognition of specific substrates by the receptor (Mangelsdorf *et al.,* 1995). The cysteine-rich central domain is responsible for binding to the DNA and is referred to as the DNA-binding domain (DBD). Eight cysteines within the DBD are organized into two zinc fingers, found in many regulatory proteins, and are responsible for DNA binding (Klug and Schwabe, 1995). NRs control gene transcription by being activated through the binding of ligands, turning the receptor “on,” allowing the small lipophilic receptor to enter the nucleus and bind to the response elements of specific target genes. Studies have shown that each nuclear receptor binds to its unique consensus response element with a certain arrangement. For example, the classic steroid receptors, including the glucocorticoid, androgen, and progesterone receptors, bind as homodimers to response elements that are composed of two hexad nucleotide sequences, configured as palindromes separated by three base

pairs (Beato, 1991). The nonsteroid receptors, including retinoic acid, vitamin D, and thyroid hormone receptors, bind to response elements, configured of two half sites, hexad length nucleotide sequences consisting of tandem repeats with differencing spacing between the half sites (Koenig *et al*., 1987; Näär *et al.,* 1991; Umesono *et al.,* 1991). The spacings between the two half sites for the response elements of vitamin D, thyroid hormone, and retinoic acid are 3, 4, and 5, respectively (this is known as the 3-4-5 rule) (Perlmann *et al*., 1993; Umesono *et al.,* 1991).

The non-steroid receptors also differ from the steroid receptors in that they bind to DNA as a heterodimer, sharing the common partner of retinoid X receptor (RXR) (Yu *et al*., 1991; Zhang *et al*., 1992). RXR is known to exist as three isoforms (RXRα, RXRβ, and RXRγ), with at least one isoform expressed in every cell of the body. The three RXR isoforms are highly conserved and are interchangeable both as receptors for 9-*cis* retinoic acid and as heterodimer partners for other NRs (Mangelsdorf *et al.,* 1992). RXR also heterodimerizes with orphan receptors that include the PPARs, LXR, FXR, PXR, and CAR. In most cases, the RXR heterodimers are ligand dependent and will only bind to response elements and activate gene transcription when bound by their respective ligands (Evans and Mangelsdorf, 2014). These RXR heterodimers play an important role in the regulation of xenobiotics and endogenous substrates. Among others, PPARs, LXR, and PXR are known to induce DMEs, including the P450s and UGTs.

Purpose of Study

Previously, we have generated the humanized *UGT1A* mouse line on a null mouse *Ugt1* background by crossing transgenic mice encoding the entire human *UGT1A* locus (Chen et al., 2005) with *Ugt1A* knockout mice (*Tg(UGT1^{A1*1})Ugt$^{-/-}$*) (Nguyen et al., 2008). By utilizing this mouse line, our goal is to understand how nuclear receptors PXR and LXRα regulate the human *UGT1* locus. We propose that the activation of these nuclear receptors plays an important role in regulating the human *UGT1* locus as well as other DMEs. To accomplish this goal, this study is divided into two parts: 1) the creation of the transgenic mouse model using bacterial artificial chromosome (BAC) transgenesis. Using this transgenic mouse line, we ultimately created the humanized PXR mouse line (*Tg(UGT1^{A1*1})Ugt$^{-/-}$Tg(Pxr)Pxr$^{-/-}$ (hUGT1*1/hPxr))* by crossing it with the *Tg(UGT1^{A1*1})Ugt$^{-/-}$Pxr$^{-/-}$ (hUGT1*1/Pxr$^{-/-}$)* mouse line that was previously developed in the lab; and 2) the regulation of the *UGT1A* locus, specifically *UGT1A1,* by activation of LXRα. We carried out a study in hUGT1A mice to examine expression of UGT1A by the LXRα ligand. The work is in progress to create the humanized UGT mouse line into an LXRα-null background to study the role of LXRα in the regulation of UGT1A locus and its impact on bilirubin metabolism, especially at the developmental stage.

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CHAPTER 2:

Creation of humanized UGT/PXR mouse model to study drug metabolism and neonatal jaundice

INTRODUCTION

The pregnane X receptor (PXR, NR1I2) is a member of the nuclear receptor superfamily and regulates gene expression by acting as a transcription factor in response to its ligands (Kliewer *et al.,* 1998). Upon the ligand binding, PXR translocates from the cytoplasm into the nucleus, heterodimerizes with RXR, and binds to DNA response elements, leading to the transcription of target genes (Squires *et al*., 2004). Studies have revealed that PXR plays an important role in the metabolism of xenobiotics and endogenous compounds, protecting organisms from toxic compounds. PXR is expressed in a broad range of tissues and is highly expressed in the liver, GI tract, and kidneys, with lower levels expressed in the uterus, brain, adrenal glands, bone marrow, ovary, placenta, and regions of the brain (Pollock *et al*., 2007; Lamba *et al.,* 2004). PXR executes its metabolic role through the induction of DMEs, including P450s and UGTs. Activation of PXR has been shown to lead to the induction of multiple P450 genes, including human *CYP3A4* and its orthologous gene in mice, *Cyp3a1*1 (Lehmann *et al*., 1998; Kishida *et al*., 2008; Drocourt *et al*., 2001). CYP3A4 is the most abundant P450 in the liver and is responsible for the metabolism of over 50% of drugs (Guengerich, 1999). Studies have also revealed that activation of PXR leads to the induction of Phase II enzymes including UGT1A1, UGT1A3, UGT1A4, and UGT1A6 (Xie *et al*., 2003; Bock, 2010). These UGTs isoforms are responsible for the metabolism of many pharmaceutical drugs including

lamotrigine, olanzapine, retigabine, irinotecan (and its metabolite SN38), acetaminophen, and nicotine. PXR activation also plays a role in the metabolism of carcinogens such as 4-nitrophenol and benzo[a]pyrene, as well as endogenous compounds such as bilirubin and steroids (Xie *et al*., 2003; Bock, 2010).

Human PXR (hPXR) activation leads to the induction of DMEs through numerous xenobiotics that are its ligands, including pharmaceutical drugs such as rifampicin (RIF), rifaximin, clotrimazole, dexamethasone, lovastatin, and metyrapone (Ma *et al.,* 2007; Goodwin *et al*, 1999; Lehmnann *et al.,* 1998)**.** PXR is also activated by various environmental pollutants and medicinal compounds derived from herbs (Coumoul *et al*., 2002; Chang, 2009). The ligand-dependent activation of PXR appears to be species specific. For example, RIF has been demonstrated to be a potent activator of human PXR but does not activate mouse or rat PXR. In contrast, PCN does not activate human PXR but is a potent activator of mouse and rat PXR (Xie *et al*., 2000). Due to the species-specific activation of PXR, using wild-type mouse models to evaluate human drug metabolism and drug-drug interactions can be problematic.

Our lab has previously developed a humanized *UGT1* (*hUGT1*1)* mouse line, which expresses the entire human *UGT1A* locus in a murine *Ugt1*-null background (Chen *et al*, 2005; Fujiwara *et al*., 2010; Nguyen *et al.,* 2008**).** Using this mouse line, we have demonstrated that expression of UGT1A

genes is tissue-specific, and they are subject to the regulation of xenobiotic receptors and during the developmental stage. As a consequence, *hUGT1*1* neonates exhibited hyperbilirubinemia with undetectable expression of UGT1A1 (Fujiwara *et al.,* 2010). These mice were crossed into a *PXR*-null background and the resulting mouse line demonstrated that the knockout of the PXR gene leads to limited induction of liver *UGT1A* genes during pregnancy. However, an induction of hepatic UGT1A1 in neonatal mice, correlated with a reduction of bilirubin levels, indicating a potential inhibitory role of PXR in *UGT1A1* expression during the developmental stage (Chen *et al.*, 2012). In this study, a human PXR transgenic mouse model was created by using a bacterial artificial chromosome (BAC) clone RP11-169N13 through transgenesis. The BAC clone contains the entire human *PXR* gene sequence (~36,000 bp), with an additional ~57,000 bp upstream of exon 1 and ~72,000 bp downstream of exon 9. The human PXR transgenic line was crossed with the mouse line, *Tg(UGT1^{A1*1})Ugt$^{-/-}$Pxr$^{-/-}$ (hUGT1*1/Pxr$^{-/-}$)* that was previously developed in the lab to create a *Tg(UGT1^{A1*1})Ugt$^{-/-}$Tg(Pxr)Pxr$^{-/-}$ (hUGT1*1/hPxr)* mouse line (*hUGT1A/hPXR*). The *hUGT1A/hPXR* mouse line can serve as a useful animal model to study the role of hPXR activation in the regulation of human *UGT1* locus *in vivo,* which may have an impact on neonatal jaundice and xenobiotic metabolism.

MATERIALS AND METHODS

Reagents

Rifampicin was purchased from Sigma-Aldrich Chemical Corporation (St. Louis, MO).

Generation of Transgenic PXR Mice

The BAC clone RP11-169N13 (BACPAC Resources) that consists of the human genomic sequence of chromosome 3 from 119,443,790 to 119,608,883 bp was used to create the transgenic mouse model. The BAC clone contains the entire human *PXR* gene sequence (~36,000 bp), with an additional ~ 57,000 bp upstream of exon 1 and ~72,000 bp downstream of exon 9. The BAC clone was verified to contain the entire human PXR gene, including the transcribed and untranscribed sequences of the *hPXR* gene and its flanking sequences, by polymerase chain reaction (PCR) amplification. Initially, the BAC clone DNA was isolated using a Maxi Prep kit (Qiagen) and a low yield of the BAC DNA was produced. We then adopted the method of equilibrium centrifugation in CsCl-ethidium bromide gradients to purify the BAC DNA. Briefly, for every mL of DNA solution, 1 g of solid CsCl was added and the solution was centrifuged at 85,000 rpm for 16 hours in a TI-100 centrifuge. The DNA bands were collected and the ethidium bromide was removed using 1-butanol saturated with water. The concentration and purity of the BAC DNA were determined by using a UV-spectrometer and agarose gel electrophoresis. The DNA was run on a 0.6% agarose gel for 60 mins at 100

volts and dialyzed against the injection buffer (10mM Tris, pH 7.5, 0.1 mM EDTA, 100 mM NaCl, 30 μM spermine, and 70 μM spermidine). The purified BAC DNA was injected into the pronucleus of $CB6F_1$ (an F1 hybrid mouse line between BALB/c and C57BL/6N mice) mouse eggs and transplanted into the oviduct of pseudopregnant C57BL/6N mice. The procedures for the generation of *hPXR* transgenic mice were completed at the Transgenic Core Facility of the University of California, San Diego. Forty mice were obtained from the Core; their tails were clipped in order to isolate DNA; and genotyping was conducted by PCR to identify the presence of the human *PXR* transgene in these mice. Mice that were positive were bred in a C57BL/6 background for two generations and then bred in a *Tg(UGT1^{A1*1})Ugt1$^{-/-}$Pxr$^{-/-}$ (hUGT1*1/Pxr$^{-/-}$)* background to generate a *Tg(UGT1^{A1*1})Ugt1$^{-/-}$Tg(Pxr)Pxr$^{-/-}$ (hUGT1*1/hPXR)* mouse line.

Animals Studies with human PXR Ligand Rifampicin

Animal studies were performed in compliance with the National Institutes of Health guidelines regarding the use of laboratory mice. Animals were maintained under controlled temperature (23°C) and lighting (12-hour light/12-hour dark cycles) and were provided with food and water ad libitum. For oral treatments of mice lines 9 and 28, two littermates, one that contains the *hPXR* gene and the other WT, were treated with RIF dissolved in PBS at a dose of 40 mg/kg through an oral route once a day for two days. Mice were sacrificed 24 hours after the last dose, and their livers, small intestines, and

large intestines were collected and stored at -80°C until RNA was isolated. In addition, *Tg(UGT1$A1^{*1}$)Ugt$^{+/-}$Tg(Pxr)Pxr$^{+/-}$* males were generated, pooled into two groups, and intraperitoneally (IP) treated with RIF (15 mg/kg) or vehicle corn oil every 24 hours for 2 days. Mice were sacrificed 24 hours after the last dose, and the liver and small intestine were collected and stored at -80°C until RNA was isolated.

PCR Genotyping

The incorporation of the BAC clone containing the human *PXR* transgene in the mouse genome was determined using multiple primers. Exon 1 (Ex1) Fwd, 5'-TCACCAGGGCTGGATTAAAG-3' and Ex1 Rev, 5'-GCCTCTGGCAACAGTAAAGC-3' amplify a 208 bp DNA product, approximately700 bp upstream of Exon 1 of the *hPXR* gene. Exon 5 (Ex5) Fwd, 5'-TCCGGAAAGATCTGTGCTCT-3' and Ex5 Rev, 5'-GCTGATGATGCCTTTGAACA-3' amplify a 164-bp DNA fragment located in exon 5 of the *hPXR* gene. Exon 9 (Ex9) Fwd, 5'-CTGTGTGGATGCTGAGCTGT-3' and Ex9 Rev, 5'-TGATTGTCAGCGTAGCCTTG-3' amplify a 214 bp DNA fragment, approximately 200 bp downstream of exon 9. For the 5' end of the BAC clone (5'-E), Fwd, 5'-GCCACTTGGCCACATACTTT-3' and 5'-E Rev, 5'-CAGCCTCCCTTTCCTTTACC-3' amplify a 334 bp sequence near the 5' end of the BAC clone. For the 3' end of the BAC clone (3'-E), Fwd, 5'-TCATCCCATCTTTCCTTTGC-3' and 3'-E Rev, 5'-

GTGGGCATCACGGTTCTACT-3' amplify a 460 bp sequence near the 3' end of the BAC clone. For the promoter region (PR), Fwd, 5'-CCACCACCTGGCTAATTT-3' and PR Rev, 5'-TTCCTGGTTTCCTTTCTTCG-3' amplify a 591 bp sequence, approximately 6500 bp upstream of exon 1.

The presence of the *hUGT1*1* transgene, mouse UGT1 knockout, and mouse PXR knockout was determined using specific primers and reaction conditions shown as follows: For the *hUGT1*1* transgene (a 490 bp product), the primers, hUGT-Fwd, 5'-TGTTGAACAATATGTCTTTGGTCTA-3' and hUGT-Rev, 5'-CCAATGAAGACCATGTTGGGC-3', were used with a PCR reaction: 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min for 35 cycles. For genotyping the *Ugt1*$^{-/-}$ mice, the primers, Ugt1$^{-/-}$-Fwd, 5′-GGGCATCTGACATGGAAAA-3′ and Ugt1$^{-/-}$-Rev, 5′-TGTAAGACAATCTTCTCCTCT-3′, were used with a PCR reaction: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min for 35 cycles to produce a 2000 bp product identified as the *Ugt1*$^{-/-}$ genotype, a 1000 bp as the *Ugt1*$^{+/+}$ genotype, or both 1000 bp and 2000 bp products as the *Ugt1*$^{+/-}$ genotype. For the knockout PXR background, three primers, WX48, 5'-AGAAACACATAGAAACCCATCCATG-3'; WX47, 5'-AGTCCACCAAGCCTG AGCCTCCTAC-3'; and WXNEO, 5'-CTTGACGAGTTCTTCTGAGGGGATC-3', were used with a PCR reaction: 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min for 38 cycles to amplify a 472 bp product identified as the WT genotype or a 521 bp product as the knockout genotype.

Measurement of CYP3A4 Gene Expression by quantitative PCR

The activity of the human *PXR* gene in the transgenic mouse lines was determined by administration of RIF, a ligand that activates human PXR but not mouse PXR, and analyzing induction of CYP3A11. For RNA analysis, tissues were frozen in liquid nitrogen and then pulverized into a fine powder. Approximately 50 mg of the powder was added to 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) to extract the RNA. cDNA was created from 1 µg of total RNA using an iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR (qPCR) was used to determine mRNA levels of m*Cyp3a11*. The qPCR reactions were performed using the SYBR Green PCR master mix (Bio-Rad) in a CFX96 Real-Time System C1000 Touch Thermal Cycler (Bio-Rad). For analysis of mouse Cyp3a11, the primers mCYP3A11 Fwd, 5'-CCGATGTTCTTAGACACTGCC-3' and mCYP3A11 Rev, 5'-CTCAATGGTGTGTATATCCCC-3" were used. The qPCR reaction was carried out at 95°C for 5 minutes, 95°C for 15 seconds, and 60°C for 20 seconds for 40 cycles, followed by 72°C for 30 seconds. Induction values were calculated using the comparative threshold cycle (Ct) method with the cyclophilin (CPH) as a control. For analysis for CPH mRNA levels, the forward primer was 5'-ATGGTCAACCCCACCGTGT-3', and reverse primer was 5'-TTCTTGCTGTCTTTGGAACTTTGTC-3'.

RESULTS

Generation of Humanized PXR Transgenic Mice

A transgenic mouse line containing the human PXR was created using theRP11-169N13 BAC clone that consists of the human *PXR* genomic sequence. The integrity of the BAC clone was verified by PCR amplification of translated and untranslated regions including a 208 bp product, approximately 700 bp downstream of exon 1 (Ex1); a 164 bp product within exon 5 (Ex5); a 214 bp product, approximately 200 bp downstream of exon 9 (Ex9); a 334 bp product near the 5'-end of the BAC clone (5'-E); a 460 bp product near the 3'-end of the BAC clone (3'-E); and a 591 bp product, approximately 6500 bp upstream of exon 1 (PR). Taken together, the BAC clone was verified to contain the expected sequences of the human *PXR* gene (Figure 2.1). The BAC clone DNA was then purified by equilibrium centrifugation in CsCl-ethidium bromide (Figure 2.2), and its purity and integrity were visualized by agarose gel electrophoresis (Figure 2.3). The gel was also used to confirm the concentration of the BAC clone DNA sample to be approximately 20 µg/mL. The BAC DNA (60 µg) was dialyzed against the injection buffer, injected into the pronucleus of $CB6F_1$ mouse eggs, and transplanted into the oviduct of pseudopregnant C57BL/6N mice.

Forty mice were obtained from the procedure performed by the Core. The mice were genotyped for integration of the complete BAC clone, including the *hPXR* transgene. The mice were initially screened for the PCR products

Ex1 and 5'-E (Figure 2.4). The results indicated that mouse lines 9, 12, 14, 18, and 28 contained at least partial integration of the *hPXR* gene. These lines were further screened to determine if they contained the entire transgene. The PCR results indicated that the mouse lines 9, 14, and 18 contained a fragmented integration of the BAC clone. Line 9 contained all exonic sequences and the PR sequence but did not contain the sequences near the 5' or 3' ends of the BAC clone. Line 14 contained the sequences near the 5' and 3' ends of the BAC clone but did not contain any exonic sequences. Line 18 contained Ex9, 5'-E, and 3'-E sequences but was missing Ex1 and PR sequences. Lines 12 and 28 contained a complete BAC clone integration, including the entire *hPXR* gene (Fig. 2.5). Therefore, lines 9, 12, and 28 were bred into a C57BL/6 background for two generations. The mice positive for the *hPXR* gene were genotyped for the complete BAC clone in preparation to cross mice into a *Tg(UGT1^{A1*1})Ugt1$^{-/-}$Pxr$^{-/-}$ (hUGT1*1/Pxr$^{-/-}$)* background to generate a *Tg(UGT1^{A1*1})Ugt$^{-/-}$Tg(Pxr)Pxr$^{-/-}$ (hUGT1*1/hPxr)* mouse line (Figure 2.6).

CYP3A11 Expression in PXR Transgenic mice

The functionality of the *hPXR* transgene in mouse lines #9 and #28 was determined by administration of RIF and evaluation of *Cpy3a11* expression through qPCR. WT mice and mice positive for the *hPXR* transgene were treated with RIF (40 mg/kg) by oral gavage for two days. The liver, small intestine, and large intestine were collected 24 hours after the last treatment,

and these tissues were pulverized under liquid nitrogen and RNA isolated. *Cyp3a11* expression levels were induced 8-fold in the liver and 10-fold in the small intestine in line #28 in response to RIF treatment compared with the control mice, indicating the *hPXR* transgene was functional (Fig. 2.7a). Similarly, expression of the *Cpy3a11* was induced 4- and 3-fold in the liver and small intestine, respectively, in line #9, indicating that line #28 has a higher response than line #9 for *Cyp3a11* expression in response to RIF treatment (Fig. 2.7b). Based on these results and line #9 containing a partial integration of the BAC clone, line #9 breeding was discontinued and lines #28 and #12 were bred into a *hUGT1*1/Pxr*$^{-/-}$ background to generate a *hUGT1*1/PXR* mouse line. Treatments were not performed on #12 due to the founder of this line dying after one litter and only two mice from the litter containing the transgene.

To insure the functionality of the *hPXR* transgene in mouse line #28, *Cyp3a11* induction by RIF was determined after one crossing into a *hUGT1*1/Pxr*$^{-/-}$ background. *Tg(UGT1*^{A1*1}*)Ugt*$^{+/-}$*Tg(Pxr*^{28}*)Pxr*$^{+/-}$ males were treated with RIF (15 mg/kg) or vehicle (corn oil) by IP injection for two days, and *Cyp3a11* gene transcripts from the liver and small intestine were identified by qPCR. In mice treated with RIF, there was an 11-fold induction of Cyp3a11 in the liver and marginal induction in the small intestine (Fig. 2.8), indicating that the transgene hPXR is functional. During the process that the *hPXR* transgene was bred in a *hUGT1*1/Pxr*$^{-/-}$ background, it was observed that,

over multiple crossings, all males were positive and all females negative for the transgene; therefore, it was determined that the transgene had incorporated into the Y chromosome. The *Tg(UGT1^{A1*1})Ugt$^{+/-}$Tg(PXR^{28})Pxr$^{+/-}$* males were crossed back into *hUGT1*1/Pxr$^{-/-}$* females to obtain the *hUGT1*1/PXR^{28}* mouse line.

DISCUSSION

The use of the BAC clone to create mice expressing human genes has proved to be an effective method to create animal models to study the regulation of DMEs, including *UGT1A* genes, in our lab. Previously, BAC clones have been used in the lab to create the human *UGT1A* transgenic mouse model (Chen *et al.,* 2005*)*, which was subsequently crossed into a murine *Ugt1a*-null background to create a humanized UGT1 (*hUGT1*1)* mouse model. The *hUGT1*1* mouse model has proven to be a valuable tool in studying neonatal jaundice and drug metabolism, including anti-tumor drug irinotecan**.** The *hUGT1*1* mouse model exhibits similar tissue-specific expression of the UGT1 proteins found in humans and displays neonatal jaundice with bilirubin levels ranging from 8-14 mg/dL, mimicking a clinical condition in some of the human infants (Fujiwara *et al.*, 2010). As a result, this animal model with hyperbilirubinemia is proven to be a valuable tool in monitoring the regulation of the *UGT1A1* because bilirubin is solely metabolized by the UGT1A1. For example, when the *hUGT1*1* mice were

crossed into a *Pxr*-null background, the absence of the Pxr resulted in an induction of hepatic UGT1A1 in neonatal mice, leading to a reduction of bilirubin levels to approximately 5 mg/dL (Chen *et al.*, 2012).

In this study, we describe the generation and characterization of the hPXR transgenic animal model. When mice were treated with RIF, a human PXR activator, hepatic Cyp3a11 expression was induced, indicating that the *hPXR* gene is functional. Interestingly, when *hPXR* transgenic mice were administered RIF orally, Cyp3a11 induction occurred in both the liver and small intestine; however, IP administration of RIF only produced Cyp3a11 induction in the liver. We further bred mouse lines #12 and #28 in a *hUGT1*1/Pxr*$^{-/-}$ background to create a humanized UGT/PXR mouse line. During the process of crossing two mouse lines, it was discovered that all males from the #28 founder line contained the h*PXR* transgene, while the transgene was absent in all females, indicating the integration of the BAC clone on the Y chromosome during implantation. The mouse model containing the *hPXR* transgene will have some limitations in that all males will be positive and all females will be negative, creating impossibility for including control mice with both male and female genders. However, we found it more convenient for genotyping because positive samples occur in male mice only. Mouse line #12, which is not sex specific, is still being crossed back into the *hUGT1*1/Pxr*$^{-/-}$ background to create the *hUGT1*1/hPXR*^{12} mouse line, the creation of which is a slower process due to breeding problems.

During the process of creating the transgenic mouse model containing the *hPXR* gene, we encountered a few technical problems. The stock *E. coli* containing the BAC clone was no longer vital at a point during the cloning process and had to be salvaged by growing the bacteria in LB broth that did not contain the respective antibiotic. After 48 hours, the *E. coli* were transferred to a medium that did contain the antibiotic and normal growth was observed. There were issues in obtaining enough of the isolated BAC clone DNA for injection into the pseudopregnant mice. The column provided in the DNA isolation kit proved to be ineffective at elucidating the DNA, and the classical technique of CsCl-ethidium bromide binding, yielding a higher DNA product, proved to be more effective. The most challenging element of preparing the isolated DNA for injection was avoidance of DNA shearing because the size of the BAC clone DNA was over 150 kb in length, making it fragile and easy to be sheared. If DNA is sheared, it can lead to partial integration into the mouse genome, as only two founders contain a complete integration and 3 founders had a partial integration. Therefore, procedures involving transferring and mixing were carried out in a very gentle manner. There is also the general problem that the BAC clone may not integrate into any of the mouse genomes. Lastly, there can sometimes be challenges crossing the transgene into the desired mouse background with general issues including poor breeders, death of the founder, multiple crossings, and the DNA cross-linking problem, in order to obtain a more complicated genetic

background (e.g., multiple human transgenes). In our case in this study, the #12 line has had issues due to the death of the founder after producing1 litter and the F1 generation being unproductive breeders.

In conclusion, using BAC clones to create mouse models expressing human genes is an effective way to study human genes in a relevant physiology condition. Since human PXR and mouse PXR possess marked differences in their ability to bind to various ligands, the *hUGT1*1/hPXR* model may be proven to be an important research tool in the study of human drug metabolism and UGT1A1-dependent neonatal jaundice.

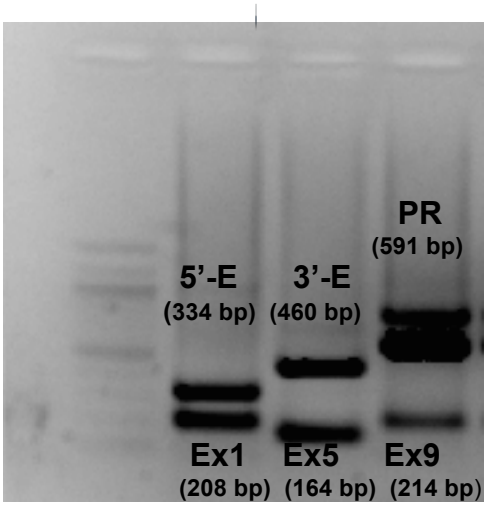


Figure 2.1. PCR amplification of *hPXR* sequences within the BAC clone. The BAC clone was verified to contain all the appropriate sequence: 208 bp product ~700 bp upstream of exon 1 (Ex1), a 164 bp product within exon 5 (Ex5), a 214 bp product sequence ~200 bp downstream of exon 9 (Ex9), a 334 bp product near the 5'-end of the BAC clone (5'-E), a 460 bp product near the 3'-end of the BAC clone (3'-E), and a 591 bp product ~6500 bp upstream of exon 1 (PR).

A B

Figure 2.2. Images of CsCl-ethidium bromide DNA binding. The BAC clone DNA was separated into four balanced Beckman centrifuge tubes and centrifuged at 85,000 rpm for 16 hours in a TI-100 centrifuge. The DNA was contained in three small bands and RNA was collected at the bottom of test tube. A, image of DNA bands under UV-light after centrifugation. B, photo of DNA bands after centrifugation taken with a flash.

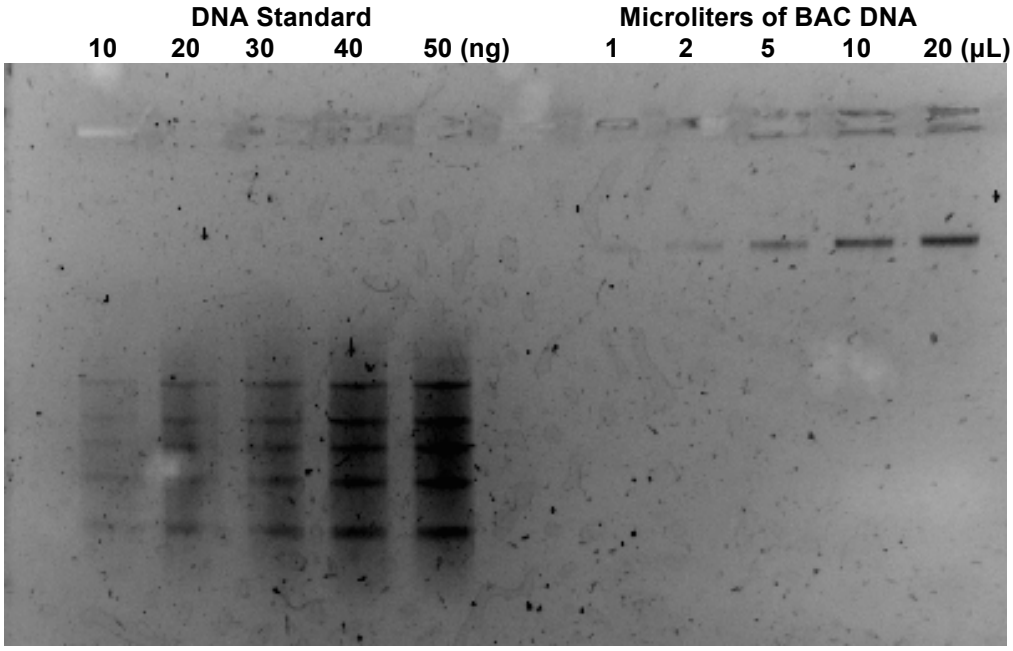


Figure 2.3. Concentration and purity of BAC clone DNA before injection. The dialyzed BAC clone DNA was ran on a 0.6% agarose gel to determine the concentration and purity. The BAC clone DNA was injected at 1, 2, 5, 10, and 20 µL against standard concentrations of DNA of 10, 20, 30, 40, and 50 ng per band. The concentration of the BAC clone DNA was determined to be ~20 µg/mL. The DNA was intact and absent of any RNA.

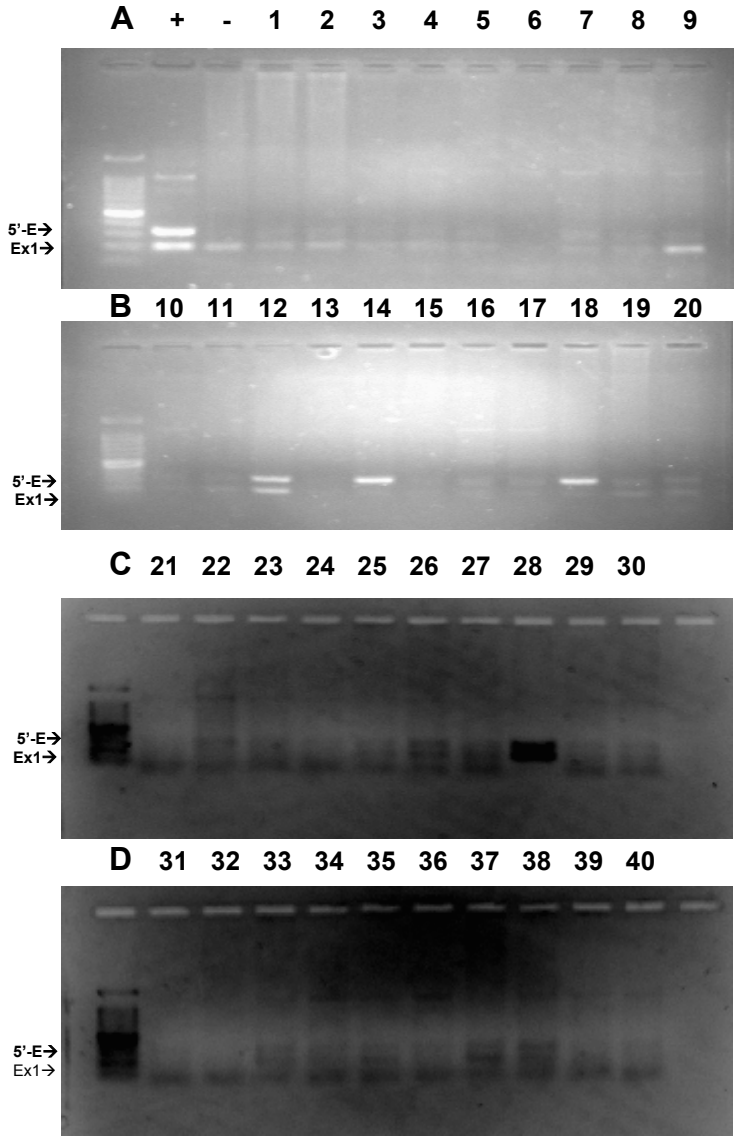


Figure 2.4. Genotyping results for *hPXR* transgenic mice. The forty mouse-tails obtained from the core were genotyped for the 5'-E and Ex1 sequences to determine if the BAC clone is integrated into the mouse genome. Mouse lines 9, 12, 14, 18, and 28 were determined to contain at least a partial integration of the BAC clone.

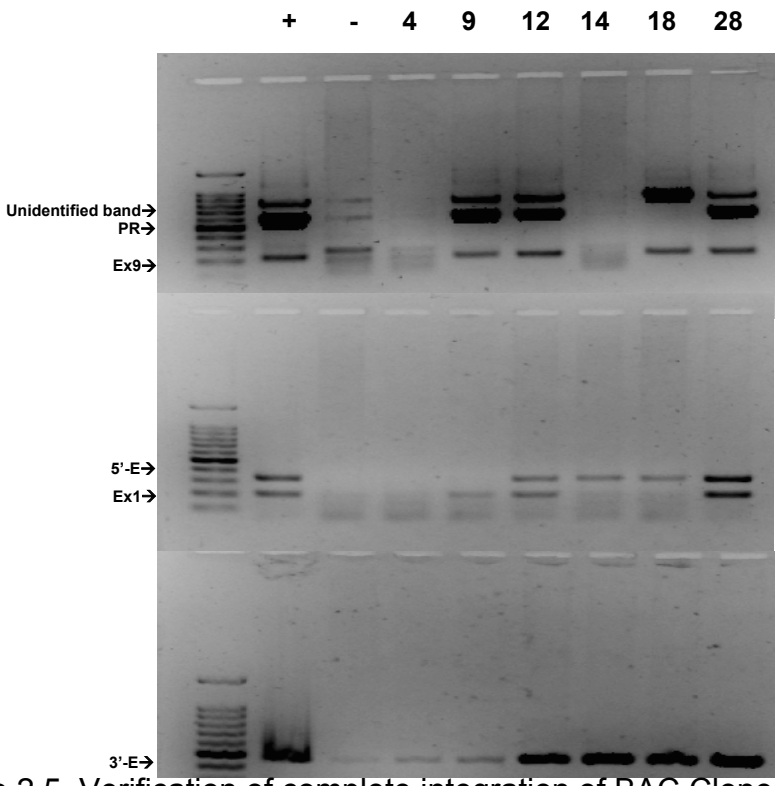


Figure 2.5. Verification of complete integration of BAC Clone. The integration of BAC clone in lines 4, 9, 12, 14, 18, and 28, along with a positive and negative control was determined by PCR amplification of specific regions within the BAC clone. Lines 18 and 28 had a complete integration of the BAC clone into the mouse genome and line 9 had a partial integration of the BAC clone containing the entire *hPXR* gene.

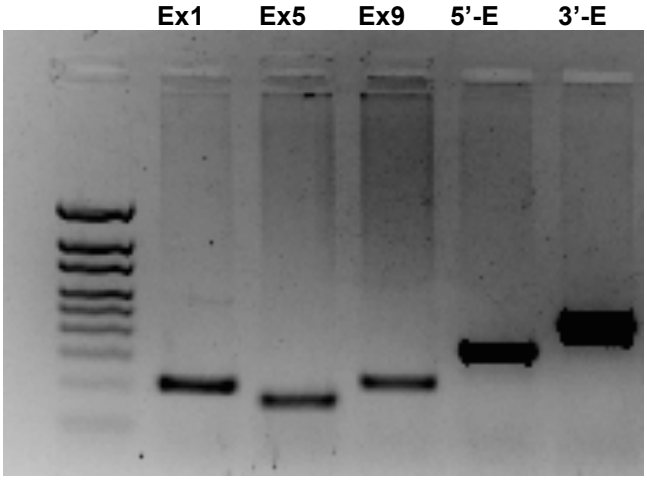
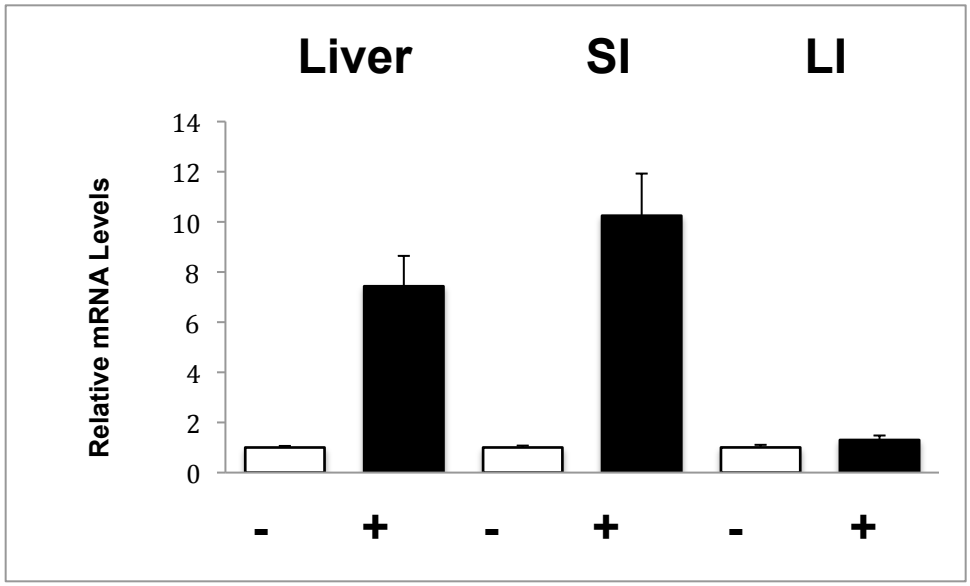


Figure 2.6. PCR genotyping of *hPXR* transgene after crossing into C57BL/6 mice for two generations. A representative genotyping for the integration of the BAC clone in lines 12 and 28 after crossing *hPXR* transgenic mice into C57BL/6 for two generations. All expected sequences were present.

A. Relative mRNA Levels of mCyp3a11 Line #28



B. Relative mRNA Levels of mCyp3a11 Line # 9

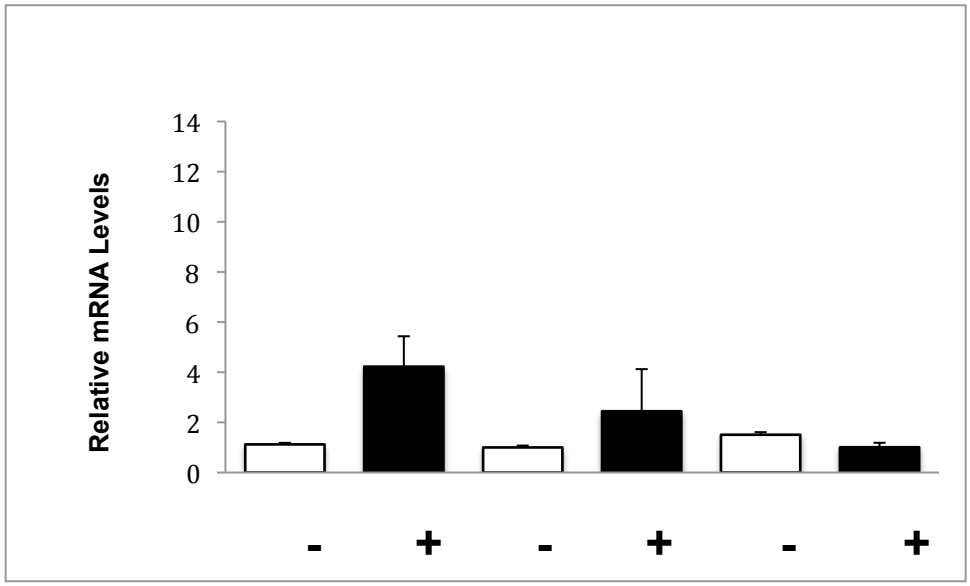


Figure 2.7. Analysis of *Cyp3a11* gene transcripts from transgenic lines treated with rifampicin. WT and mice positive for the human PXR transgene were treated with rifampicin (40 mg/kg) by oral gavage for two days, and Cyp3a11 gene transcripts were identified by qPCR. Values were quantified using comparative CT method with samples normalized to CPH. The values are expressed as fold-change and control mice (vehicle treatment) were expressed as 1-fold. A, Cyp3a11 expression of line # 28. B, Cyp3a11 expression of line # 9.

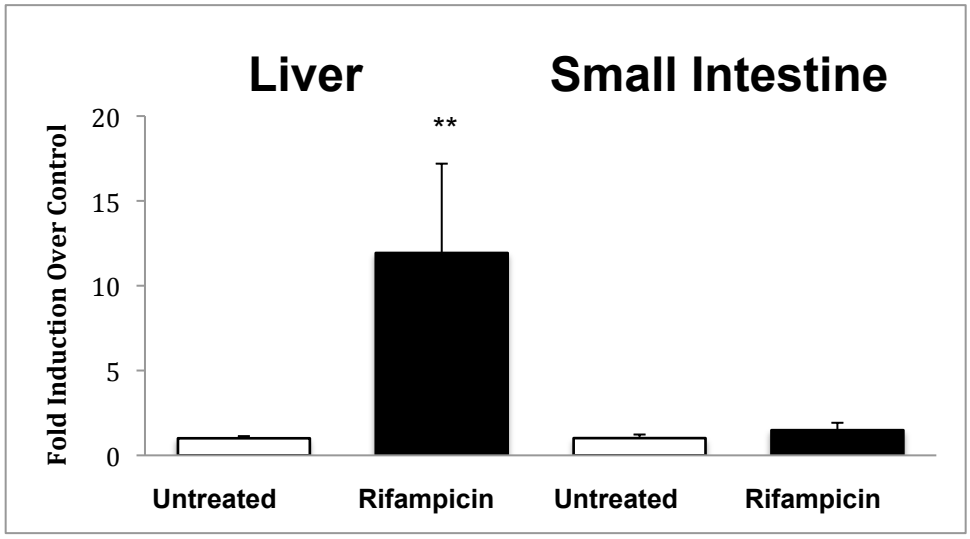


Figure 2.8. Analysis of *Cyp3a11* gene transcripts from transgenic line #28 treated with rifampicin. *Tg(UGT1^{A1*1})Ugt1$^{+/-}$Tg(PXR)Pxr$^{+/-}$* males were treated with rifampicin (15 mg/kg) by IP injection for two days, and Cyp3a11 gene transcripts from liver and small intestine were identified by qPCR. Animals treated with corn oil were labeled as untreated. Values were quantified using the comparative CT method with samples normalized to CPH. Data represent the mean ± SD (n=2). Statistically significant differences between control and treated mice are indicated by asterisks (Student t test: **: $p < 0.01$)

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CHAPTER 3:

Induction of UGT1A1 by LXR agonist T0901317

INTRODUCTION

The liver X receptor (LXR), originally classified as an orphan receptor until the discovery of its endogenous ligand oxysterols, is a member of the nuclear receptor superfamily (Janowski *et al.,* 1996). Two isoforms of LXR have been discovered in humans, LXRα (NR1H3) and LXRβ (NR1H2), and they are located on chromosomes 11p11.2 and 19q13.3, respectively (Willy *et al*., 1995; Teboul *et al.,* 1995). LXRs are a ligand-activated transcription factors and control gene expression by forming heterodimers with RXR, which then binds to specific DNA sequences known as responsive elements in the promoter regions of the target genes (Willy *et al.,* 1995). LXR activation has been demonstrated to be involved in either activating or repressing target genes, which is determined by the presence of the co-activator or co-repressor complexes (Desvergne *et al.,* 2006) including nuclear receptor co-repressor (NcoR) and silencing mediator of retinoic acid and thyroid hormone receptors (SMRT). LXRβ is ubiquitously expressed across all tissues (Song *et al.,* 1994); in contrast, LXRα expression is typically restricted to the liver, intestines, kidney, spleen, adipose tissue, and macrophages (Willy *et al.,* 1995).

LXRs were initially discovered as regulators of cholesterol homeostasis and have also been linked to other major physiological functions (Peet *et al.,* 1998) including glucose homeostasis (Cha *et al*., 2007), lipid metabolism (Volle et al., 2004), steroidogenesis, immunity, and inflammation (Zelcer et al., 2006). Studies in LXR knockout mice have demonstrated the pathogenesis of

diseases that include atherosclerosis, obesity, diabetes, Alzheimer's disease, cancer, and decreased fertility in both males and females (Huang, 2014). The wide range of maladies that are present in LXR knockout mice indicates that the LXR agonist may be a significant pharmacological agent. However, LXR agonists currently have not been proven to be effective at treating LXR-related diseases because synthetic ligands caused adverse side effects, including increased triglycerides, in animals (Huag, 2014). Studying the role of LRX in the regulation of their target genes may help the understanding of its potential to be a therapeutic target.

Our lab has previously developed a humanized *UGT1*1* (*hUGT1*1)* mouse line, which expresses the entire human *UGT1* locus in a murine *Ugt1a* null background (Chen et al, 2005; Fujiwara et al., 2010; Nguyen et al., 2008). These mice have demonstrated a tissue-specific expression of *UGT1* genes, which are also subject to the regulation of xenobiotic receptors. These mice had undetectable UGT1A1 levels and exhibited hyperbilirubinemia during neonatal development (Fujiwara et al., 2010). Using the hUGT1*1 mouse line, we have previously demonstrated that LXR activation leads to UGT1A3 induction (Verreault et al., 2006). In the current study, the regulation of the *UGT1* locus, specifically *UGT1A1,* by activation of LXR was examined. Mice administered the LXR agonist, T090137, showed an induction of the UGT1A1 in multiple tissues and reduction in bilirubin levels. These observations prompted us to breed *hUGT1*1* mice into an *LXRα-null* background to further

study the interaction of LXR and the *UGT1* locus.

MATERIALS AND METHODS

Reagents

T0901317 was purchased from Cayman Chemical company (Ann Arbor, Michigan), Tween 80 was purchased from Sigma-Aldrich (St. Louis, MO), and propylene glycol was purchased from J.T. Baker (Phillipsburg, NJ).

Animal studies with LXR agonist T090137

Animal studies were performed in compliance with the National Institutes of Health guidelines regarding the use of laboratory mice. Animals were maintained under controlled temperature (23°C) and lighting (12-hour light/12-hour dark cycles) and were provided with food and water ad libitum. Mice were pooled and divided into two groups, treatment and controlled groups (n=4). T090137, a known LXR agonist, was dissolved in propylene glycol/tween 80 (4:1) and was orally administered once to mice at the dose of 80 mg/kg, and control mice were administered the vehicle. Mice were sacrificed 48 hours after the dose and liver, small intestine, and large intestine were collected, pooled into respective groups, and stored at -80°C until RNA was isolated. For serum total bilirubin analysis, blood was collected from mice at time of treatment, 24 hours, and 48 hours after treatment by puncture of the submandibular vein. The blood was centrifuged at 2,000 x g for 5 minutes and bilirubin levels measured using a Unistat Bilirubinometer (Reichert, Inc.)

Measurement of *UGT1A1* Gene Expression by qPCR

For RNA analysis, tissues from the same groups were pooled, frozen in liquid nitrogen, and then pulverized into a fine powder. Approximately 50 mg of the powder was added to 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) to extract the RNA. cDNA was created from 1 µg of total RNA using a iScript cDNA Synthesis Kit (Bio-Rad). qPCR was used to quantitate UGT1A1 mRNA expression. The qPCR reactions were performed using SYBR Green PCR master mix (Bio-Rad) in a CFX96 Real-Time System C1000 Touch Thermal Cycler (Bio-Rad). For analysis of human UGT1A1 RNA, the forward primer 5'-AACAAGGAGCTCATGGCCTCC-3' and the reverse primer 5'-GTTCGCAAGATTCGATGGTCG-3' were used, and a qPCR reaction was carried out at 95°C for 5 minutes, 95°C for 15 seconds, and 60°C for 20 seconds for 40 cycles, followed by 72°C for 30 seconds. Induction values were calculated using the Ct method with CPH as an internal control. For analysis for CPH RNA, the forward primer was 5'-ATGGTCAACCCCACCGTGT-3', and reverse primer was 5'-TTCTTGCTGTCTTTGGAACTTTGTC-3'.

RESULTS

Oral administration of T090137 Induces UGT1A1 in *hUGT1*1* Mice

To discover the potential of LXRα to induce human *UGT1A* gene expression in vivo, *hUGT1*1* mice were treated with the LXR agonist T090137 (80 mg/kg) once by oral gavage. Mice were sacrificed 48 hours after the

dosing, and the tissues were collected and pooled (four per treatment group). After pulverization of liver, small intestine, and large intestine tissues with liquid nitrogen, RNA was prepared from these tissues. qPCR with UGT1A1-specific primers was conducted to compare UGT1A1 expression between treatment and control groups. In mice treated with T090137, there was a prominent induction of UGT1A1 in the liver (800-fold) and a milder but still significant induction in small intestine (12-fold) and large intestine (8-fold) (Fig. 3.1).

The bilirubin levels of the mice were monitored during treatment to verify the induction of UGT1A1. Bilirubin is a by-product of heme and solely conjugated by UGT1A1, allowing bilirubin monitoring to be an effective means of tracking UGT1A1 expression. Blood samples were taken at the time of treatment (mice were 13 days old at the start of treatment), 24 hours after treatment, and 48 after treatment when tissues were collected. Twenty-four hours after mice were treated with T090137, there was a 50% reduction in bilirubin from ~10 mg/dL to ~5 mg/dL, and bilirubin levels were reduced to below 1 mg/dL 48 hours after mice were treated with T090137, indicating that UGT1A1 gene induction may be through LXR (Fig. 3.2). Based on these results, *the hUGT1*1* mice were crossed into an *LXRα-null* ($LXR^{-/-}$) mouse background to further determine the role of LXR in regulating UGT1A1 expression and altering bilirubin metabolism.

DISCUSSION

This study potentially identifies the human *UGT1A1* as a target gene for LXRα. After administration of T0901317 to *hUGT1*1* mice, there was an approximately 800-fold, 11-fold, and 8-fold induction in the liver, small intestine, and large intestine, respectively, compared with vehicle-treated mice. The induction of UGT1A1 was further supported by the reduction of serum total bilirubin levels from 10 mg/dL to below 1 mg/dL 48 hours after treatment. We also measured expression of other UGT1A isoforms in the liver, small intestine, and large intestine (not reported). Based on these preliminary results, the *hUGT1*1* mouse line was crossed into an *LXRα-null* background to create a *hUGT1*1/LXRα*$^{-/-}$ mouse line to further study the involvement of LXRα in the regulation of the UGT1A locus and in other biological functions. In the process of creating this mouse line to date, all *hUGT1*1/LXRα*$^{-/-}$ mice generated died before 21 days of age. It is hypothesized that the combination of knocking out LXRα with the *hUGT1*1* mice is lethal due to hyperbilirubinemia with extremely high levels of bilirubin. One *hUGT1*1/LXRα*$^{-/-}$ mouse exhibited the total bilirubin level at 26 mg/dL. Typically, extremely high levels of bilirubin exceeding 20 mg/dL are lethal to neonates due to central nervous system damage. We are in the process of investigating this hypothesis; if confirmed, it would be the first instance where the knockout of a nuclear receptor in combination with the *hUGT1*1* gene locus is lethal, revealing that LXRα plays an essential role in the regulation of neonatal

jaundice and drug metabolism.

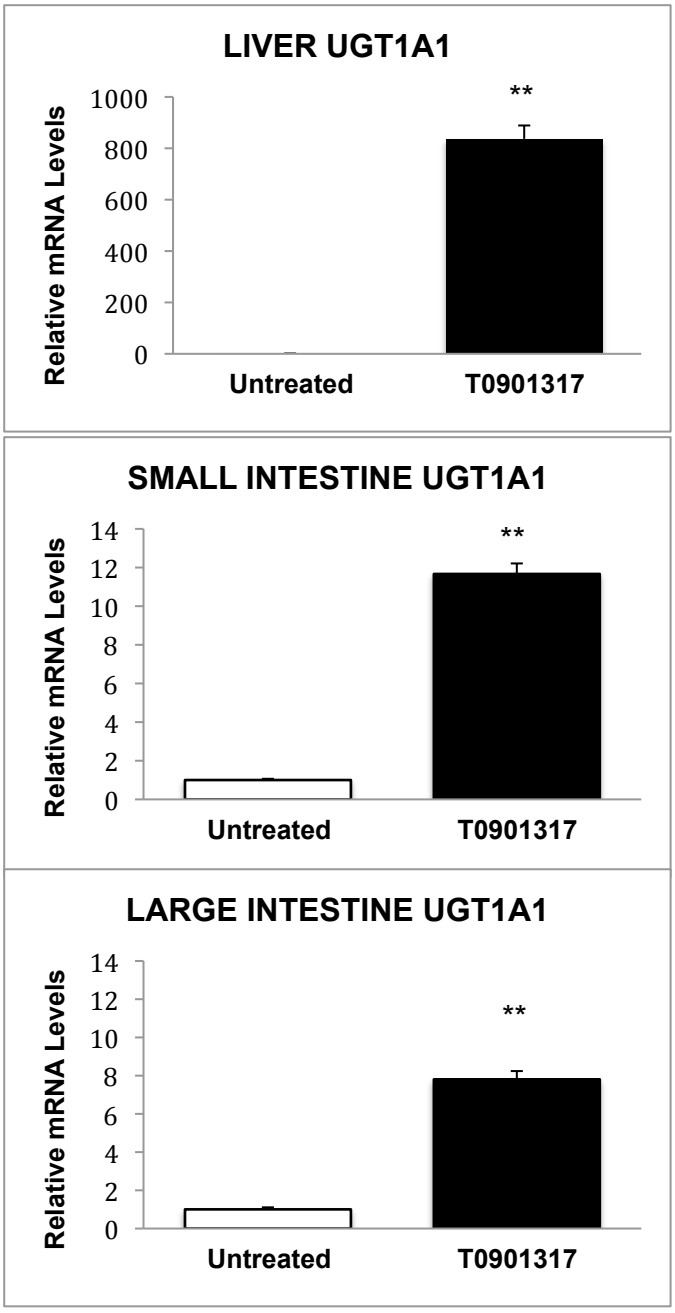

Figure 3.1. Induction of UGT1A1 in T0901317-treated hUGT1*1 mice. hUGT1*1 mice were treated with T0901317 (80mg/kg) by oral gavage and induction UGT1A1 gene transcripts were identified by qPCR. For these experiments, tissues were pooled before RNA isolation. Data represents the mean ± SD (n=4). Statistically significant differences between control and treated mice are indicated by asterisks (Student t test: **: $p < 0.0001$)

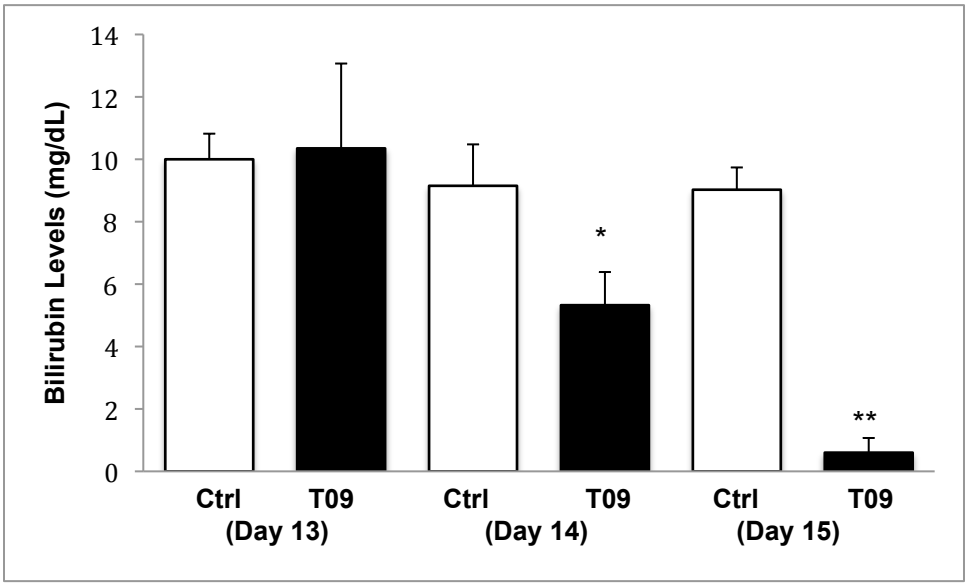

Figure 3.2. Serum total bilirubin during treatment with T0901317 in hUGT1*1 mice. Blood samples were taken at the time of treatment (mice were 13 days old at the start of treatment), 24 hours after treatment, and 48 hours after treatment when tissues were collected. Data represents the mean ± SD (n=4). Statistically significant differences between control and treated mice are indicated by asterisks (Student t test: **: $p < 0.0001$; *: $p < 0.005$)

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