THE ROLE OF EPIGENETICS IN TRANSCRIPTIONAL REGULATION OF FXR AND SILENCING FXR EXPRESSION IN HUMAN COLON CANCER

By

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Submitted to the graduate degree program in Toxicology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Farnesoid X receptor (FXR) is a ligand activated transcription factor belonging to the nuclear receptor superfamily and bile acids are its endogenous ligands. FXR is a critical regulator of the enterohepatic circulation of bile acids, lipid homeostasis, glucose metabolism, and tumor suppression in liver and intestine. Consequently, FXR has become a very promising therapeutic target for the prevention and/or treatment of cholestasis, hyperlipidemic disorders, metabolic syndrome, and liver and colon cancer. Studies suggest epigenetic mechanisms are critical for proper transcriptional induction of nuclear receptors. Likewise, evidence shows epigenetic mechanisms are responsible for modulating the tissue/cell-specific FXR expression in human colon cancer. However, how epigenetic mechanisms are involved in FXR induced transcription or tissue-specific FXR expression remains elusive. Understanding these mechanisms is crucial for future development of pharmacological modulators of FXR as well as understanding the full physiological roles of FXR. This dissertation was designed to elucidate epigenetic mechanisms involved in tissue-specific FXR induced gene transcription, orphan nuclear receptors critical for regulating FXR function, and epigenetic mechanisms responsible for FXR silencing in colon cancer. In specific aim 1, a genome-wide FXR binding assay was done in mouse liver and intestine. Specific aim 2 focuses on the role of the orphan nuclear receptor hepatocyte nuclear factor 4 alpha (HNF4 α) in regulating liver-specific functions of FXR. And finally, in specific aim 3, DNA methylation of FXR promoter was investigated as the mechanism responsible for FXR silencing in human colon cancer. In conclusion, genome-wide binding of FXR implicates novel epigenetic mechanisms and orphan nuclear receptors in regulating FXR function. Furthermore, this study indicates

that HNF4α is at least one orphan nuclear receptor capable of regulating FXR function in the liver. Findings from these first two aims succeeded in progressing drug development fields aimed at finding new FXR modulators for the treatment of multiple metabolic disorders by elucidating novel epigenetic mechanisms that may be investigated as therapeutic targets. Finally, FXR is at least partially down-regulated by DNA methylation in human colon cancer, suggesting a potential mechanism to be targeted for the prevention, treatment, and/or diagnosis of colon cancer.

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Finally, but certainly not least, I would like to thank God. I firmly believe my success in graduate school and in life has not been by my efforts alone, but a blessing and gift from God. At the risk of sounding overly dramatic, I strongly believe pursuing a doctoral degree in science was something I was directed towards and it has taken nothing short of divine intervention for me to complete. And for this, I am eternally grateful.

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List of Abbreviations

APC: adenomatous polyposis coli

APOA-I: apolipoprotein A-I

APOC-II: apolipoprotein C-II

APOC-III: apolipoprotein C-III

ASBT: apical sodium dependent bile salt transporter

AZA: azacytidine

BAAT: bile acid-CoA: amino acid N-acyltransferase

BSEP: bile salt export pump

CA: cholic acid

CAR: constitutive androstane receptor

CARM-1: coactivator associated arginine methyl transferase-1

CDCA: chenodeoxycholic acid

ChIA-PET: chromatin interaction analysis with paired-end tag sequencing

ChIP: chromatin immunoprecipitation

ChIP-qPCR: chromatin immunoprecipitation followed by quantitative PCR

ChIP-Seq: chromatin immunoprecipitation followed by massive parallel sequencing

Co-IP: co-immunoprecipitation

COL1A2: collagen-α2

CYP7A1: cytochrome p450 7A1; cholesterol 7 alpha-hydroxylase

CYP8B1: cytochrome p450 8B1; sterol 12-alpha-hydroxylase

CYP3A4: cytochrome p450 3A4

DBD: DNA binding domain

DCA: deoxycholic acid

DNMT: DNA methyltransferase

DR-1: direct repeat separated by one nucleotide

ER-2: everted repeat separated by two nucleotides

ERα: estrogen receptor alpha

FGF 15/19: fibroblast growth factor 15/19

FGFR4: fibroblast growth factor receptor 4

FOXA1: foxhead box protein A1

FXR: farnesoid X receptor

FXRRE: FXR response element

H3K4: histone 3 lysine 4

H3K4me: histone 3 lysine 4 monomethylation

H3K9: histone 3 lysine 9

H4K20: histone 4 lysine 20

H3K27: histone 3 lysine 27

H3K36: histone 3 lysine 36

HAT: histone acetyltransferases

HCC: hepatocellular carcinoma

HNF4α: hepatocyte nuclear factor alpha

IBABP: ileal bile acid binding protein

ICP: intrahepatic cholestasis of pregnancy

IGB: integrated genome browser

IP: immunoprecipitation

IR-1: inverted repeat separated by one nucleotide

L-FABP: liver fatty acid binding protein

LBD: ligand binding domain

LCA: lithocholic acid

LPL: lipoprotein lipases

LRH-1: liver receptor homolog 1

MACS: model-based analysis of ChIP-seq

MeDIP: methylated DNA immunoprecipitation

mEH: microsomal epoxide hydrolase

MEME: multiple em for motif elicitation

MRP2: multidrug resistance protein 2

NAFLD: non-alcoholic fatty liver disease

NCOR: nuclear corepressor

NFκB: nuclear factor kappa B

NR0B2: nuclear receptor subfamily 0, group B, member 2

NR1H4: nuclear receptor subfamily 1, group H, member 4

NR1I2: nuclear receptor subfamily 1, group I, member 2

NTCP: Na/taurocholate cotransporting polypeptide

OATP: organic anion transporting polypeptides

OST α: organic solute transporter alpha

OST β: organic solute transporter beta

PCR: polymerase chain reaction

PGC1α: peroxisome-proliferator-receptor (PPAR)-gamma-coactivator-1α

PRMT-1: protein arginine methyl transferase-1

PXR: pregnane X receptor

RXRα: retinoic X receptor alpha

RNA Pol II: RNA polymerase II

SHP: small heterodimer protein

SMRT: silencing mediator of retinoic acid and thyroid hormone receptor

SRC-1: coactivators steroid receptor coactivators 1

SREBP-1c: sterol regulatory element-binding protein 1c

TG: triglyceride

TF: transcription factor

TNFα: tumor necrosis factor alpha

TSS: transcription start site

UTR: untranslated region

WNT: wingless-int

Chapter 1: Introduction and Background

1.1: Introduction to Farnesoid X Receptor (FXR):

Farnesoid X Receptor (FXR) is a ligand activated nuclear receptor belonging to the nuclear receptor superfamily. FXR was first identified in yeast as an orphan nuclear receptor capable of heterodimerizing with retinoid X receptor alpha (RXRα) and transcriptionally responding to RXR ligands (Seol et al., 1995). A mouse homologue of FXR was subsequently cloned and found to respond to supraphysiological concentrations of farnesol, an intermediate of the mevalonate biosynthetic pathway, whereby it obtained its name (Forman et al., 1995). Shortly after being cloned, bile acids were identified as endogenous ligands of FXR, and therefore, FXR became an adopted orphan nuclear receptor (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999).

The FXR protein is encoded by the gene *NR1H4* and is highly expressed in the liver, intestine, kidney, and adrenal gland, with low expression being found in the heart, and adipose tissues (Zhang et al., 2003). The FXR protein exhibits the classical nuclear receptor structure homology consisting of a highly conserved DNA binding domain (DBD), a C-terminal region containing a ligand binding domain (LBD) and a ligand-dependent transactivation domain, AF2, and a ligand-independent transactivation domain, AF1, at the N-terminus (**Figure 1**; Modica et al., 2010).

The DNA binding domain contains two Zn^{2+} finger motifs needed for DNA binding and dimerization with RXRα (reviewed by Olefsky, 2001; Pellicciari et al., 2005). The ligand binding domain contains a hydrophobic pocket critical for the binding of bile acids.

Figure 1: The classical protein structure of FXR.

The AF2 and AF1 domains are responsible for mediating interactions with co-regulatory proteins (reviewed by Modica et al., 2010).

FXR is classified as a type II nuclear receptor which are different from type I, III, and IV nuclear receptors by localizing mainly to the nucleus and initiating gene transcription predominantly as a heterodimer with RXRα. Type II nuclear receptors consist mainly of subfamily 1 members of the nuclear receptor superfamily. Despite FXR's classification as a type II nuclear receptor, whether nuclear translocation of FXR occurs upon ligand binding is not clear. In the best accepted scenario, FXR constitutively sits on FXR response elements (FXRREs) within target genes as a heterodimer with RXRα. In the absence of bile acids, the FXR-RXRα dimer interacts with corepressors and represses gene transcription. Upon binding of bile acids, the FXR-RXRα heterodimer releases corepressors and recruits coactivators, and subsequently initiates transcription of FXR target genes (reviewed by Modica et al., 2010). In an alternative and less characterized scenario, FXR is sequestered either in the cytosol or nucleus, or both, and binding of bile acids induces access of FXR to the chromatin, heterodimerization with RXRα, enrichment of FXR to FXRREs located in target genes, recruitment of coactivators, and initiation of target gene transcription. Existing evidence suggests that FXR may function in both of these manners (Chong et al., 2010; Fang et al., 2008; Rizzo et al., 2005; Thomas et al., 2010). Nevertheless, the most well characterized FXRRE is an inverted repeat (A/GGG/TCA) separated by one nucleotide (IR-1).

As mentioned, FXR is highly expressed in liver and intestine and its main characterized role is regulating the enterohepatic circulation of bile acids. However,

recent studies have elucidated roles of FXR in lipid and glucose metabolism and have suggested FXR as a potential therapeutic target for the treatment of cholestasis, hyperlipidemia, type II diabetes, non-alcoholic fatty liver disease (NAFLD), and gastrointestinal cancers (**Figure 2**). FXR's role in all of these processes will be reviewed below.

1.1.1: Role of FXR in the enterohepatic circulation of bile acids.

The original generation of FXR knockout (KO) mice was associated with elevated serum bile acid levels and changes in gene expression of genes involved in bile acids synthesis, transport, and metabolism revealing the significant role FXR plays in regulating bile acid homeostasis (Kok et al., 2003; Sinal et al., 2000). Since then, FXR has been determined to be a master regulator of the enterohepatic circulation of bile acids (**Figure 3**). To begin, bile acids are synthesized in the liver through the enzymatic conversion of cholesterol to primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA). There are multiple enzymatic pathways that convert cholesterol to primary bile acids within different hepatic cellular compartments. However, the main bile acid synthesis pathway includes the enzymatic conversion of cholesterol to equal ratio of CA and CDCA by the rate-limiting enzyme CYP7A1, located on the endoplasmic reticulum membrane, and is termed the "classical pathway" (Russell, 2003). Primary bile acids are further metabolized by conjugation to glycine or taurine (Russell and Setchell, 1992; Sjovall, 1959; Vlahcevic et al., 1999). Bile acids are mainly transported out of the liver and into the bile duct through the ATP-dependent bile salt export pump (BSEP;

Figure 2: FXR and disease. FXR has been shown to attenuate the development of several diseases (listed below). Therefore, FXR has become a potential therapeutic target for the treatment of these diseases.

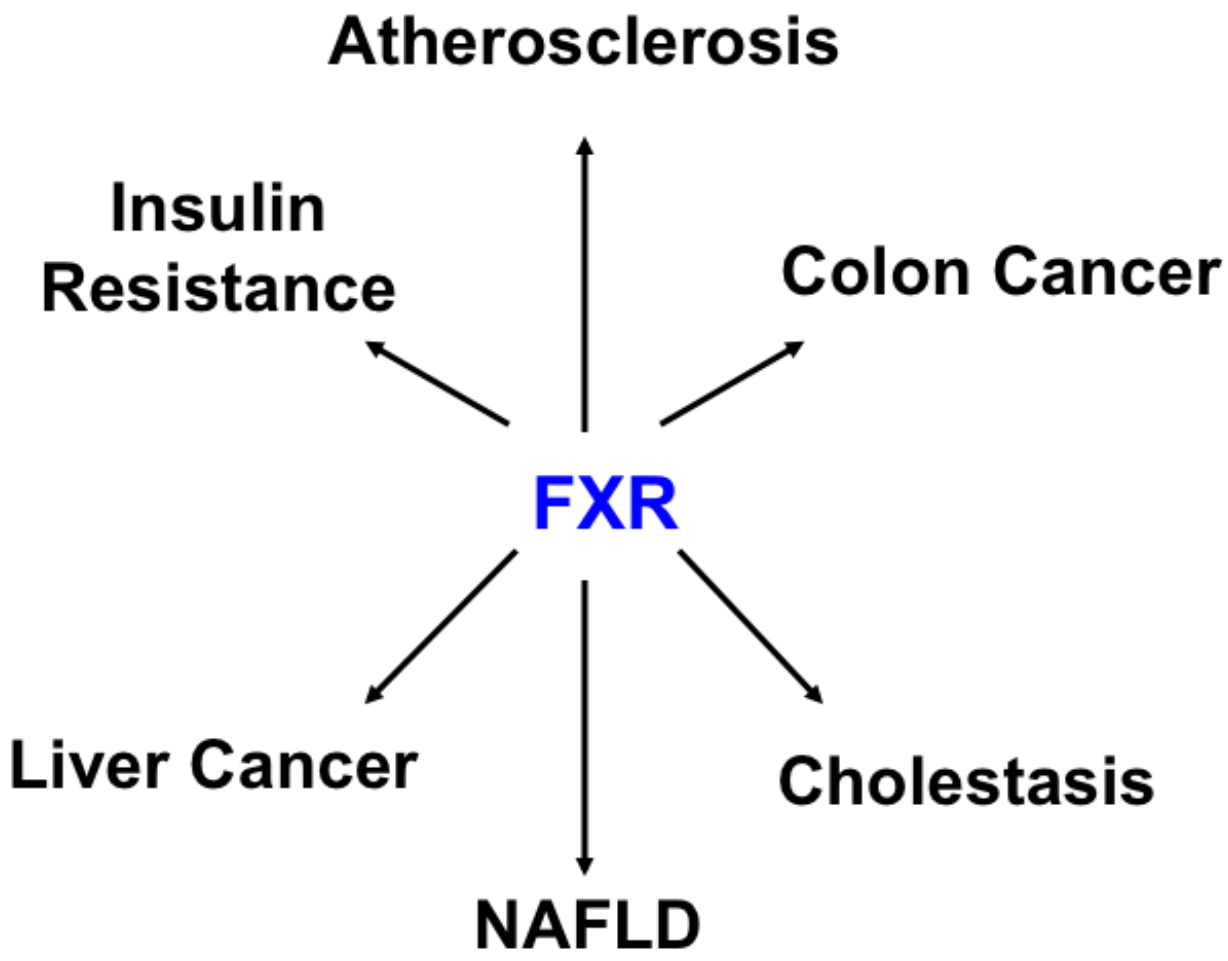
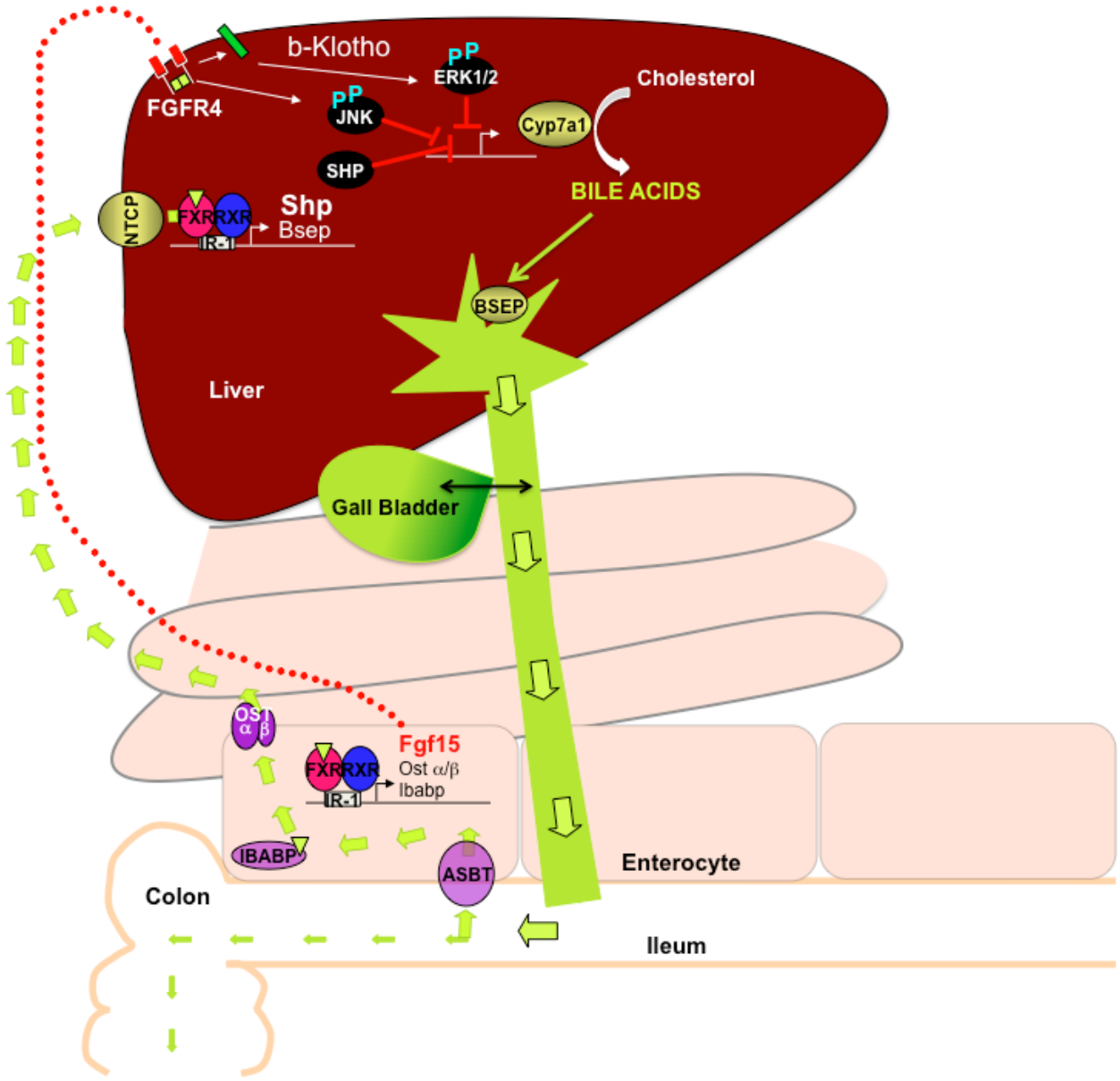


Figure 3: Enterohepatic circulation of bile acids (for detailed description, see text).



Gerloff et al., 1998; Noe et al., 2002). Di-anionic conjugated bile acids are not substrates for BSEP and can be effluxed through the cannalicular multidrug resistance protein 2 (MRP2; Keppler et al., 1997; St-Pierre et al., 2001).

Bile acids flow from the bile duct into the gall bladder where they are stored. After a meal, the endocrine cells of the intestinal mucosa release cholecystokinin stimulating the gall bladder to contract and releasing bile acids into the intestinal lumen of the duodenum (Hofmann, 1999). Bile acids aid in the emulsification and absorption of ingested lipids and lipid soluble vitamins in the small intestine and are reabsorbed via active transport in the distal ileum (Ridlon et al., 2006). Enterocyte uptake of conjugated bile acids is mediated via the apical sodium dependent bile salt transporter (ASBT; Geyer et al., 2006; Shneider et al., 1995; Wong et al., 1994). As bile acids enter enterocytes of the ileum, they are retrieved intracellularly by the ileal bile acid binding protein (IBABP; Kramer et al., 2001; Trauner and Boyer, 2003). IBABP is involved in intracellular trafficking of bile acids by retrieving them from the apical membrane and delivering them to either nuclear compartment or basolateral membrane. IBABP is suggested to help prevent bile acid induced toxicity to intestinal cells (Fang et al., 2007). Bile acids can bind to and activate intracellular FXR within enterocytes of the distal ileum. FXR then initiates transcription of target genes by forming a homodimer or heterodimer with RXRα and binding DNA response elements within regulatory regions of response genes.

Intestinal activation of FXR induces the expression of fibroblast growth factor (FGF) 15 in mice, or FGF19 in humans, IBABP, and efflux transporters organic solute transporters (OST) α and β (Dawson et al., 2005; Grober et al., 1999; Inagaki et al.,

2005; Landrier et al., 2006). OSTα and OSTβ heterodimerize at the basolateral membrane of enterocytes and efflux bile acids from enterocytes into the portal circulation (Dawson et al., 2005; Landrier et al., 2006). Nearly 95% of the primary bile acids are absorbed by ileal enterocytes via passive diffusion or facilitated and active transport. However, 5% of conjugated primary bile acids continue in route towards the colon where colonic bacteria can metabolize primary bile acids to the secondary bile acids, lithocholic acid (LCA) and deoxycholic acid (DCA). DCA is hydrophobic and can be readily reabsorbed by colonic epithelium by passive diffusion and/or active transport via similar mechanisms described above, and is transported back to the liver. DCA is mainly reabsorbed and accumulates in the enterohepatic bile acid pool where as LCA is excreted in the feces (Cowen et al., 1975; Ridlon et al., 2006).

FGF15/19 is synthesized in the ileum of the small intestine and is a secreted peptide that travels via the portal circulation or lymphatics back to the liver (Inagaki et al., 2005). FGF15/19 selectively binds to the basolateral transmembrane protein FGF receptor 4 (FGFR4) in the liver. FGFR4 has tyrosine kinase activity and is suggested to signal through MAP kinase pathways (Xie et al., 1999). The ultimate binding of FGF15/19 to FGFR4 results in the inhibition of CYP7A1/Cyp7a1 transcriptional activation and down-regulation in bile acid synthesis (Inagaki et al., 2005).

As mentioned, bile acids absorbed in the intestines by either passive diffusion or through the uptake transporter ASBT are bound intracellularly to IBABP and then transported into portal circulation via OSTα/β (Grober et al., 1999; Kramer et al., 2001; Landrier et al., 2006). In the portal circulation, bile acids bound to plasma protein albumin are circulated back to the liver where they dissociate from albumin (Berk et al.,

1987; Roda et al., 1982) and are actively taken up into the hepatocyte mainly by the uptake transporter Na/taurocholate cotransporting polypeptide (NTCP; Hagenbuch et al., 1991; Meier, 1995; Schroeder et al., 1998). Bile acids have also been reported to be taken up into hepatocytes through sodium independent mechanisms (Kouzuki et al., 1998; Maglova et al., 1995; Yamaguchi et al., 2006) including uptake by organic anion transporting polypeptides (OATPs) 1B1 and 1B3 (Yamaguchi et al., 2006). It has also been suggested that microsomal epoxide hydrolase (mEH) mediates sodium dependent bile acid uptake (Von Dippe et al., 1993; von Dippe et al., 1996), however, the mechanism and significance of this is controversial (Hagenbuch et al., 1996). Intracellular bile acids in hepatocytes bind to liver fatty acid binding protein (L-FABP) which facilitates bile acid trafficking to the cannalicular membrane (Martin et al., 2005). Hepatic intracellular bile acids bind to and activate FXR which homodimerizes or heterodimerizes with RXRα and induces expression of FXR target genes, including small heterodimer partner (SHP) and BSEP (Goodwin et al., 2000). SHP is a universal inhibitory factor that inhibits liver receptor homolog 1 (LRH-1) induced transcription of CYP7A1 and CYP8B1 (Goodwin et al., 2000). Therefore, SHP up-regulation in the liver, in cooperation with FGF-15/19 from the intestine, results in an additive down-regulation of bile acid synthesis (Goodwin et al., 2000; Inagaki et al., 2005). In essence, bile acids reabsorbed in the intestines function to feedback inhibit synthesis of bile acids in the liver.

1.1.2: Role of FXR in lipid and glucose metabolism.

As mentioned, FXR has been shown to play a role in regulating other metabolic pathways aside for its role in bile acid homeostasis, including lipid and glucose metabolism. Studies in mice have shown that FXR activation has beneficial effects on lipid and glucose parameters within disease mouse models revealing its potential as a target for the treatment of diseases such as hyperlipidemia, fatty liver, and diabetes (**Figure 2**).

Shortly after discovering FXR as a transcriptional regulator of bile acid synthesis and metabolism, it was speculated that FXR may play a role in lipid metabolism. It was subsequently shown that mice and rats treated with a synthetic FXR agonist, GW4064, had decreased plasma triglyceride (TG) levels (Claudel et al., 2003; Maloney et al., 2000; Zhang et al., 2006). Next, FXR was shown to negatively regulate the transcription of sterol regulatory element-binding protein 1c (SREBP-1c), a transcription factor (TF) essential for stimulating fatty acid synthesis (Watanabe et al., 2004). In a mouse model of obesity and type 2 diabetes, the KK-A(y) mouse, FXR activation resulted in a reduction of liver and serum TG levels and very low density lipoprotein secretion through FXR-induced transcription of SHP (Watanabe et al., 2004). It was further elucidated that SHP can interfere directly with liver X receptor's (LXR) ability to bind to SREPB-1c promoter and induce transcription leading to an overall suppression of lipogenesis as a consequence of FXR activation (Watanabe et al., 2004). In addition, FXR can also directly modulate transcription of genes involved in TG clearance by inducing genes responsible for activating lipoprotein lipases (LPL) and TG hydrolysis, such as apolipoprotein C-II (APOC-II) (Kast et al., 2001), and repressing transcription of genes involved in LPL inhibition, such as apolipoprotein C-III (APOC-III; Claudel et al.,

2003). Therefore, FXR has become a potential therapeutic target for the treatment of hyperlipidemic disorders.

In addition, FXR's role in glucose metabolism has become a more recent field of interest when studies showed that altering bile acid profiles modified glucose parameters in models of diabetes (Staels and Kuipers, 2007) suggesting the regulation of bile acid synthesis and glucose metabolism may be linked. It was then shown that a rat model of diabetes had decreased liver expression of FXR with a correlating increase in bile acid pool size, and insulin administration returned FXR levels to normal (Duran-Sandoval et al., 2004). These results indicate glucose directly regulates the expression of FXR. Furthermore, FXR has also been shown to regulate insulin-sensitivity. This was seen in FXR KO mice which have impaired glucose-tolerance and insulin sensitivity (Cariou et al., 2006; Ma et al., 2006; Zhang et al., 2006). The effects of FXR on insulin sensitivity are not well elucidated, but have been suggested to be mediated by FXRs induction of FGF15/19 (Kir et al., 2011). However, FXR has also been shown to directly repress the transcription of rate-limiting enzymes involved in gluconeogenesis via the FXR-SHP transcriptional pathway (Ma et al., 2006; Zhang et al., 2006). Although more work is needed to further characterize the comprehensive roles of FXR in glucose metabolism, there are promising data suggesting FXR agonists as a treatment option for patients with type II diabetes.

Because FXR has emerged as a major regulator of several metabolic processes including cholesterol, bile acid, lipid and glucose metabolism, research targeting FXR as a therapeutic option for the treatment of disorders such as cholestasis, hyperlipidemia, and metabolic syndrome should be further pursued. In order to accomplish this, though,

a better understanding on how FXR induces transcription of target genes on a genome-wide and tissue-specific scale is needed.

1.1.3: Targeting FXR for pharmacotherapy.

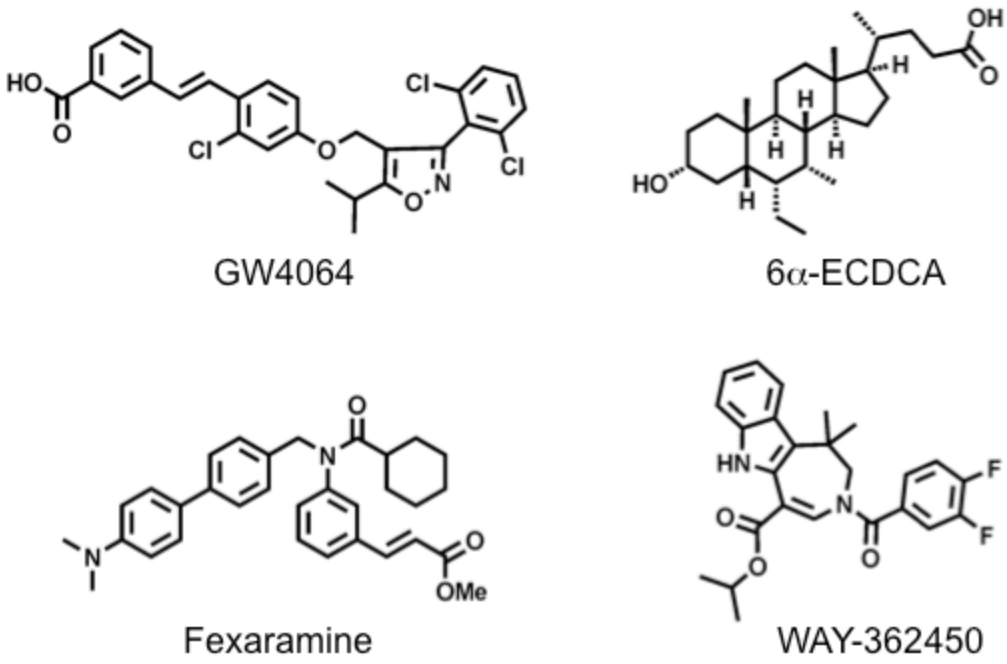
Over the last two decades, FXR's role in regulating multiple metabolic pathways has become increasingly intriguing for the drug development field to design FXR modulators for the treatment and/or prevention of several diseases. FXR's role in regulating the synthesis, transport, and metabolism of bile acids has allowed it to be a potential target for the treatment of cholestasis and hypercholesterolemia. Likewise, as was described above, FXR role in negatively regulating genes involved in lipogenesis and gluconeogenesis suggest it as a promising therapeutic target for disorders associated with metabolic syndrome. Finally, though still poorly understood, FXR has been suggested to suppress the development of colon and liver cancers (Kim et al., 2007; Modica et al., 2008; Yang et al., 2007), and therefore has become a possible target for the prevention and/or treatment for these cancers.

Using bile acids for targeting FXR as a therapeutic agent has become less desirable largely due to toxicities associated with elevated bile acid levels, as well as other adverse side effects such a pruritus, a condition involving itching of the skin due to elevated systemic bile acid levels (Varadi, 1974). Therefore, synthetic and semi-synthetic FXR agonists have been development in order to enhance selective activation of FXR (**Figure 4,** obtained from PubChem Compound). Semi-synthetic FXR modulators have been developed by chemically modifying the steroid nucleus backbone of bile acids. An example of this is a 6α-ethyl derivative of DCA (6α-ECDCA, INT-747),

which is currently in phase II clinical trials for the treatment of primary biliary cirrhosis (Intercept Pharmaceuticals, NCT00550862). In addition, stilbene derivatives of synthetic retinoids were developed for selective activation of FXR, leading to the development of GW4064 (Maloney et al., 2000), a potent and selective activator of FXR with relatively low toxicity and, unfortunately, low bioavailability (Kim et al., 2007). This led to the development of a more bioavailable FXR agonist, WAY-362450, in-licensed by Exelixis, intended for the treatment of hyperlipidemia (Evans et al., 2009; Flatt et al., 2009). Likewise, synthetic biaryl cinnamate derivates of the benzopyran backbone, such as fexaramine and fexarine, were developed as selective and potent FXR agonists (Downes et al., 2003). However, all of these synthetic FXR agonists seem to activate FXR target genes in different manners suggesting that these synthetic ligands may act as gene-selective FXR modulators (Downes et al., 2003). In addition to synthetic modulators, emerging evidence has shown natural products as being capable of activating FXR, such as cafestol and green tea catechins (Ricketts et al., 2007) unpublished data from Guo lab), which also may serve as potential therapeutic options for treatment and/or prevention of disease, namely intestinal disease such as irritable bowel disorder, ulcerative colitis, and colon cancer.

However, as we know from the use of modulators of other nuclear receptors, such as agonists for estrogen receptor and the peroxisome proliferator family of receptors, many adverse health effects can be associated with targeting nuclear receptors for the treatment of human disease. In the case of FXR, little is known about the systemic off-target effects associated with FXR activation. However, some adverse

Figure 4: Chemical structures of semi-synthetic and synthetic FXR modulators (for details see text).



effects have been noted and/or speculated. Therefore, much research is needed in understanding the genome-wide, tissue-specific, and systemic effects of FXR activation. This is necessary so that FXR modulators can be developed to enhance beneficial effects of FXR activation, while minimizing off-target effects.

1.2: The Role of Epigenetic Mechanisms in Regulating Nuclear Receptor Function:

Conrad Waddington first introduced the term ‘epigenetics’ in 1942 to describe the interaction of genes with the environment which brought about a certain phenotype. Epigenomics was later coined as the study of chromatin structure, packaging of DNA around nucleosomes, covalent modifications of histone tails, DNA methylation (Murrell et al., 2005), non-coding RNAs, particularly microRNAs (Jirtle and Skinner, 2007), as well as other mechanisms. These epigenetic mechanisms are thought to be involved in processes such as development, tissue-specific expression of genes, senescence, development of diseases, such as cancer, as well as many other processes. All of these epigenetic mechanisms function to alter the expression of genes without altering the underlying genetic information.

In order for nuclear receptors to bind to response elements in DNA and initiate transcription of target genes a permissible chromatin environment is essential to expose DNA response elements. Chromatin rearrangement can typically occur through modifications to histones comprising nucleosome cores that allow localized chromatin to transition from a close conformation to an open conformation. In the context of nuclear receptor mediated transcriptional activation, chromatin modification occurs through the

recruitment of coactivators to target gene binding sites. Therefore, in order for nuclear receptors to induce transcription of target genes, an intricate organization of cofactor recruitment and chromatin modification is necessary.

Emerging results have shown that the recruitment of nuclear receptors and cofactors to target gene regulatory regions occurs in a specific sequential order (Metivier et al., 2006). In fact, it has been suggested that in order for a nuclear receptor to bind to its response element in a target gene regulatory region binding of another factor is necessary to direct nuclear receptor binding, which referred to as a pioneer factor. Therefore, expression of a nuclear receptor in a cell type or tissue alone is not sufficient to induce transcription of target genes. The remainder of this section will discuss common histone modifications associated with active and repressed gene transcription and an overview of cofactors and pioneer factors that are involved in nuclear receptor-mediated transcriptional regulation.

1.2.1: Overview of histone modifications associated with active/repressed gene transcription.

Nucleosomes make up the basic unit of chromatin. Nucleosomes are comprised of 146 base pair strings of DNA wrapped around core histone proteins, H2A, H2B, H3 and H4, linked by a 200 base pair string of DNA between each unit (Kouzarides, 2007; Luger et al., 1997; Trotter and Archer, 2008; Wolffe, 2001). The DNA that is wrapped around the histone complex is unavailable for the binding of a TF or for the initiation of transcription. In order for a TF or nuclear receptor to bind to its response elements with the promoter or enhancer region of a target gene and initiate transcription, the tightly

wound chromatin in these regions needs to be released by unwinding allowing the binding sites to be exposed. Furthermore, chromatin needs to be further modified and unwound to allow other mediators to associate with the transcription complex, such as RNA polymerase II (RNA Pol II), and to allow optimal space for DNA-protein interactions. All of these actions are suggested to occur through the epigenetic modification of histone tails (Kouzarides, 2007). These modified histones then alter their conformation in such a way that they free bound DNA in order for transcription to occur.

There are multiple posttranscriptional modifications that can occur to tails of histones 1-9 (H1-H9). Some of the more significant histone modifications are: methylation of arginines; methylation, acetylation, ubiquitination, ADP-ribosylation, and sumoylation of lysines (K); and phosphorylation of serines and threonines (Bernstein et al., 2007; Kouzarides, 2007). How each modification alters the histone-DNA complexes and what role these modifications have on transcription, DNA-replication, DNA repair or other mechanisms is not fully known. However, recent studies have begun to elucidate some of these roles (Kouzarides, 2007). For example, in the instance of transcription initiation instigated by binding of a TF, it has been shown that mono-, di- and tri-methylation of histone 3 lysine 4 (H3K4) and lysine 36 (H3K36) is highly associated with actively transcribed genes (Bernstein et al., 2007; Heintzman et al., 2007; Li et al., 2007). Conversely, methylation of H3 lysine 9 (H3K9), H3 lysine 27 (H3K27), and H4 lysine 20 (H4K20) correlates with gene repression (Bernstein et al., 2007). Acetylation of histone lysines causes a neutralization of charge resulting in relaxation in the nucleosome core, and therefore is almost always associated with active transcription (Bernstein et al., 2007; Kouzarides, 2007). Activation of nuclear receptors leads to

recruitment of coactivators which are responsible for mediating these histone modifications. This process will be described in more detail below.

1.2.2: Overview of cofactors and pioneer factors that are involved in nuclear receptor-mediated transcriptional regulation.

As mentioned, in order for nuclear receptors to bind to response elements in DNA and initiate transcription of target genes a permissible chromatin environment is needed, which is accomplished through modifications to histones comprising nucleosome cores allowing localized chromatin to transition to an open conformation. Nuclear receptors initiate chromatin modification through the recruitment of coactivators to target gene binding sites. These coactivators include the SRC family of proteins and provide a scaffold for the recruitment of other coactivators including histone acetyltransferases (HAT) and histone methyltransferases, which covalently modify histones to allow for a permissible chromatin environment necessary for induction of nuclear receptor mediated transcription (Berger, 2007; Kinyamu and Archer, 2004; Kouzarides, 2007; Perissi and Rosenfeld, 2005; Stallcup et al., 2003). In addition to this, ATP-dependent chromatin remodeling complexes are also recruited to target gene promoters in order to further modify localized chromatin to allow for other cofactors and transcriptional machinery to bind and initiate target gene transcription (Aoyagi and Archer, 2008; Aoyagi et al., 2005).

Research is currently being conducted to analyze where nuclear receptors bind within target genes and how binding alters the structure of chromatin in order to illicit transcription. One such TF suggested to regulate this process is foxhead box protein A1

(FOXA1; hepatocyte nuclear factor 3β). FOXA1 is a pioneer factor involved in recruitment of estrogen receptor alpha (ERα) to cis-regulatory elements within target genes in order to induce transcription (Lupien et al., 2008). Studies have shown FOXA1 binding highly correlates with H3K4 di-methylation to the promoter regions of actively transcribed FOXA1 target genes (Lupien et al., 2008). In addition, FOXA1 binding is absolutely essential for the proper transcriptional activation of ER target genes (Hurtado et al., 2011; Lupien et al., 2008). Studies describing the involvement of pioneer factors and epigenetic modifications, such as H3K4 di-methylation, in regulating ERα-induced transcription of target genes implicates that FXR also initiates transcription of its target genes through associations with similar epigenetic mediators. However, which cofactors and epigenetic mediators are involved in FXR-induced transcription of target genes needs to be experimentally determined.

It has already been shown that binding of ligands to FXR induces a conformation change that releases interactions to the corepressors silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear corepressor (NCOR), and recruits the coactivators steroid receptor coactivator 1 (SRC-1), peroxisome-proliferator-receptor (PPAR)-gamma-coactivator-1α (PGC1α; Savkur et al., 2005; Zhang et al., 2004), coactivator associated arginine methyl transferase-1 (CARM-1; Ananthanarayanan et al., 2004; Bauer et al., 2002), protein arginine methyl transferase-1 (PRMT-1; Rizzo et al., 2005), and other coactivators (Modica et al., 2010). And therefore, FXR functions to initiate transcription of target genes in much the same manner as other nuclear receptors. However, knowledge of how FXR initiates transcription of target genes in a tissue-specific manner is still lacking. A few tissue-specific target genes for FXR have

already been determined. For example, even though FXR is highly expressed in liver and intestine, it only induces expression of FGF15 in intestine and BSEP in liver (Ananthanarayanan et al., 2001; Inagaki et al., 2005). Other tissue-specific factors and epigenetic mechanisms are likely responsible for regulating tissue-specific functions of FXR. Determining the epigenetic mechanisms involved in FXR-induced transcription of FXR target genes can greatly enhance our understanding of the cellular machinery involved in FXR target gene expression.

1.3: Introduction to HNF4α in regulating liver homeostasis:

Hepatocyte nuclear factor 4 alpha (HNF4α) is encoded by the gene *HNF4A* and has been shown to be a critical factor for regulating both embryonic and adult liver development (Hayhurst et al., 2001; Watt et al., 2003). HNF4α is an essential regulator of the transcription of genes involved in hepatocyte cell lineage important for liver development (Strick-Marchand and Weiss, 2002). HN4α localizes mainly to the nucleus, binds DNA exclusively as a homodimer, and recognizes response elements consisting of direct repeats, namely direct repeats separated by one nucleotide (DR-1; Bogan et al., 2000; Jiang et al., 1995; Jiang and Sladek, 1997). HNF4α regulates the transcription of several liver specific genes including genes involved in the production of clotting factors, apolipoprotein synthesis, and drug metabolism (Gonzalez, 2008; Watt et al., 2003). In addition, HNF4α has been shown to directly regulate the transcription of CYP7A1, the rate-limiting enzyme in bile acid synthesis, suggesting HNF4α also has a role in regulating bile acid homeostasis (Inoue et al., 2004). Studies have shown that HNF4α is capable of enhancing the liver-specific functions of other type II nuclear receptors. For

example, HNF4α has been shown to cooperatively enhance the transcriptional activity of constitutive androstane receptor (CAR) and pregnane X receptor (PXR) at the cytochrome p450 3A4 (CYP3A4) promoter (Tirona et al., 2003). Whether HNF4α functions to regulate FXR activity has not been determined. However, evidence exists showing correlative roles of these two factors in bile acid homeostasis and suggests that HNF4α could be an important factor involved in the regulation of tissue-specific functions of FXR.

1.4: Introduction to role of FXR in colon cancer progression:

Colon cancer is a leading cause of cancer related deaths in the United States. Although inheritable genetic factors contribute significantly, diets high in saturated fat and low in fiber comprise a major risk factor for colon cancer development (Armstrong and Doll, 1975). Dietary fats are emulsified by primary bile acids in the proximal intestines in order for proper digestion and absorption and high-fat diets can lead to an elevated intestinal bile acid load. Furthermore, low dietary fiber can increase the gastrointestinal transit time, and together, these factors can increase both the level and time of bile acid exposure (Correa, 1981; Willett et al., 1990).

In the distal ileum and colon, primary bile acids are converted to secondary bile acids by intestinal microflora. Secondary bile acids, DCA and LCA, are considered to be more cytotoxic compared to primary bile acids, CA and CDCA, due to their detergent-like properties and higher hydrophobicity (Hofmann, 1999). DCA and LCA have been shown to induce colonic epithelium cytotoxicity through oxidative stress and promote cell proliferation, and therefore, have been linked to increased colon

carcinogenesis (Lechner et al., 2002). In fact, it has been shown that patients with colorectal cancer have elevated levels of secondary bile acids in their feces (Bianchini et al., 1989; Hill et al., 1975; Reddy et al., 1978).

As mentioned earlier, FXR functions to regulate the synthesis, transport, intestinal re-absorption, and free intracellular concentration of bile acids (**Figure 3**). This process is essential for preventing the accumulation of bile acids to cytotoxic levels (Inagaki et al., 2005; Okuwaki et al., 2007; Sinal et al., 2000; Tu et al., 2000; Zollner et al., 2006). Independently of bile acids, FXR has also been shown to attenuate the development of intestinal tumors in mouse models of colon cancer (Maran et al., 2009; Modica et al., 2008). This suggests that FXR may serve as a tumor suppressor for the development of colon cancer. However, the exact mechanisms involved in FXR-induced tumor suppression are unknown. One mechanism could be through the protection of colonic epithelium from bile-acid toxicity by inducing intracellular trafficking proteins and efflux transporters while suppressing the expression of bile acid influx transporters and the *de novo* synthesis of bile acids. However, FXR appears to have anti-tumorigenic functions independent of its regulation of bile acid homeostasis. For example, FXR deficiency has been suggested to increase susceptibility to colon cancer development by increasing epithelial permeability to bacteria and promoting WNT/β-catenin signaling as a result of tumor necrosis factor alpha (TNFα) released from infiltrating macrophages (Inagaki et al., 2006; Modica et al., 2008). Likewise, FXR deficiency has also been shown to promote the development of hepatocellular carcinoma (HCC) through stimulation of WNT and nuclear factor kappa B (NFκB) signaling (Kim et al., 2007; Wang et al., 2008; Wolfe et al., 2011; Yang et al., 2007). Furthermore, constitutive

activation of FXR in colon cancer cell lines protects cells against tumorigenesis, via suppression of cellular proliferation and activation of apoptosis (Modica et al., 2008). Therefore, a bile acid-independent anti-tumorigenic role of FXR is highly likely.

FXR expression is inversely related to the malignancy of colon cancer cell lines suggesting that colon cancer cells have developed a mechanism to selectively decrease FXR expression (De Gottardi et al., 2004). Although polymorphisms present in the FXR gene have been identified and associated with decreased function in intrahepatic cholestasis of pregnancy (ICP; Marzolini et al., 2007), to date, no clinically known mutations exist within the FXR gene to explain the decrease in FXR expression or function in human colon cancer. Therefore, it has been proposed that FXR expression may be regulated by epigenetic mechanisms. DNA methylation is a common cellular mechanism responsible for suppressing gene expression during development or tumorigenesis. The purpose of this project was to elucidate whether DNA methylation of the FXR gene is responsible for FXR reduction in human colon cancer.

1.5: Introduction to DNA Methylation in Cancer:

Tumor suppressor genes are commonly down-regulated in cancer cells by DNA methylation of their promoters (Baylin et al., 1998). However, covalent DNA methylation can occur within promoter regions, introns, and/or 3' UTRs of a tumor suppressor gene. Methylation typically occurs within regions of the gene that are rich in CpG sites, or otherwise known as CpG islands (Jones and Baylin, 2002; Takai and Jones, 2002). A CpG island is defined as sequences greater than 200 bp in length, with GC content greater than 50% and an observed CpG/expect CpG ratio of 0.6 or greater (Takai and

Jones, 2002). Four active DNA methyltransferases (DNMT) have been identified in mammals, DNMT1, DNMT2, DNMT3A and DNMT3B. However, only DNMT1 and DNMT3B are mainly responsible for aberrant DNA methylation (Jones and Baylin, 2002; Rhee et al., 2002). Which one(s) of these, if any, is specifically involved in the methylation of the FXR gene is not known.

1.6: Concluding Introductory Remarks:

Over the last few decades, there has been an extensive amount of research done to elucidate the physiological roles of FXR. In 20 years, identification of FXR functions has gone from being an unknown orphan nuclear receptor capable of heterodimerizing with RXRα in yeast to being a critically important mediator of bile acid homeostasis, lipid and glucose metabolism, and protecting the integrity of the liver and intestine. This research has determined FXR as a very promising therapeutic option for multiple metabolic diseases, which is quite intriguing given the major health impact these diseases have on the United States, as well as the rest of the world. However, because FXR remains a relatively “new” member of the nuclear receptor superfamily, much is still left to understand about FXR functions, including the epigenetic mechanisms involved in FXR-induced transcription and cell-specific expression of FXR.

New technological advances have made it possible to further understand epigenetic mechanisms involved in nuclear receptor activity on a genome-wide scale. Elucidating how FXR functions on a genome-wide scale and the epigenetic mechanisms involved in regulating FXR function, will have a profound effect on developing pharmacological FXR modulators and on the field of nuclear receptor

research. In addition, understanding epigenetic mechanisms involved in regulating cell-specific expression of FXR, namely expression in hepatocytes and enterocytes, will help elucidate the role FXR plays in diseases, such as liver and colon cancer, and determine whether FXR is a potential therapeutic option for these diseases. The ultimate goal of this thesis is to further elucidate the epigenetic mechanisms involved in mediating FXR induced transcription of target genes and involved in silencing of FXR in human colon cancer.

Chapter 2: Statement of Purpose

2.1: Significance:

FXR has become a potential therapeutic target for the treatment, prevention, and/or diagnosis of several diseases, including cholestasis, fatty liver disease, hyperlipidemia, and liver and colon cancer. However, knowledge of the epigenetic mechanisms involved in FXR-mediate transcriptional regulation of target genes and in FXR silencing in colon cancer is lacking. Understanding these processes is absolutely essential for the development of FXR modulators as well as for understanding FXR's role in the etiology of these diseases. My dissertation was, therefore, designed to elucidate the epigenetic mechanisms involved in: 1) regulating genome-wide FXR binding in liver and intestine; and 2) regulating FXR expression in human colon cancer.

2.1.1: A comprehensive genome-wide view of FXR function is essential for understanding tissue-specific functions of FXR and for development of therapeutic modulators of FXR.

The best characterized role of FXR is regulating the enterohepatic circulation of bile acids. However, research over the past decade has revealed FXR also critically regulates lipid and glucose metabolism, and serves a tumor suppressive role for liver and colon cancer development. In addition to this, tissue-specific gene regulation of FXR has already been determined for some genes. For example, FXR has been determined to regulate the expression of FGF15 and ASBT in intestines but not liver (Inagaki et al., 2005; Li et al., 2005). Conversely, FXR has been shown to regulate

BSEP and NTCP in liver but not intestines (Ananthanarayanan et al., 2001; Plass et al., 2002; Zollner et al., 2005). However, the degree of FXR tissue-specificity on a genome-wide scale and the epigenetic mechanisms involved remain unknown.

Until the recent development of genome-wide binding technology, obtaining information on the direct effects of TF binding on target gene expression has been difficult. Previously, this information was obtained by microarray analysis. However, because FXR can regulate gene transcription via several methods, using microarray analysis for understanding the global effects of FXR activation on target gene expression has its limitations. For example, FXR can regulate expression of genes by directly binding to regulatory regions within DNA and either initiate or repress expression of the gene. This is in fact how FXR regulates the transcription of target genes SHP, APOC-III, and apolipoprotein A-I (APOA-I; Claudel et al., 2003; Claudel et al., 2002; Lu et al., 2000). However, FXR can also regulate gene transcription indirectly through up-regulating the transcription of other transcription modulators. For example, SHP is considered a universal inhibitory protein. Up-regulation of SHP by FXR results in the inhibition of transcription of other genes such as LRH-1, CYP7A1 and SREBP-1c (Lu et al., 2000; Watanabe et al., 2004). Finally, FXR can regulate the transcription of genes through regulation of bile acid levels. Bile acids themselves can serve as signaling molecules. For example, bile acids can bind to an activate a G-protein couple receptor, TGR5, resulting in increased intracellular cAMP levels, activation of MAP kinase signaling cascades, and induction of gene transcription, independent of its effects on FXR activity (Kawamata et al., 2003; Maruyama et al., 2002; Takeda et al., 2002). In order to fully elucidate the direct effects of FXR activity on gene expression, a

genome-wide binding analysis is necessary. In addition, genome-wide binding analysis will help elucidate the extent of FXR's role in other cellular processes, such as lipid metabolism, glucose metabolism, and tumor suppression, on a genome-wide scale.

This project was designed to investigate the genome-wide binding of FXR in liver and intestine in order to get a better understanding of genome-wide tissue-specific functions of FXR. This analysis should yield interesting information on the degree of tissue-specific binding of FXR in liver and intestine, molecular pathways regulated by FXR in liver and intestine, and will suggest some novel ideas of how FXR regulates transcription of target genes. In addition, genome-wide binding analysis of FXR will help determine the degree of binding of FXR to desirable and non-desirable target genes, which is critical for the development of FXR modulators.

2.1.2: Orphan nuclear receptors, namely HNF4α, and epigenetic mediators may help to regulate FXR function.

Information gained from genome-wide binding of FXR in liver and intestine revealed evidence of orphan nuclear receptors regulating FXR function. Much has already been discussed regarding the recruitment of chromatin modifying enzymes and cofactors involved in FXR induced transcription of target genes. However, little is known about the interaction of FXR with other orphan nuclear receptors. The significance of this is profound because of the implications on pharmacologically targeting an orphan nuclear receptor to modulate specific functions of FXR. This could be a potential therapeutic option for targeting desirable FXR activity while minimizing off-target effects. In addition, elucidating the coordination of FXR activity with other orphan nuclear

receptors adds to the overall scientific understanding of FXR function and further progresses the nuclear receptor field.

HNF4α is an orphan nuclear receptor that transcriptionally regulates several liver-specific genes. In addition, HNF4α has been shown to regulate both the synthesis and metabolism of bile acids suggesting it has overlapping function with FXR (Inoue et al., 2004). Furthermore, HNF4α has been shown to cooperatively enhance the transcriptional activity of other type II nuclear receptors in the liver, CAR and PXR (Tirona et al., 2003), and therefore, may likely facilitate liver-specific functions of FXR. Consequently, a comprehensive study on the role of HNF4α in regulating liver-specific functions of FXR on a genome-wide scale is necessary.

2.1.3: Understanding the role of FXR and the mechanism of FXR down-regulation in colon cancer could provide valuable information for targeting FXR for the prevention, treatment, and/or diagnosis of colon cancer.

Previous studies have shown that FXR expression is decreased in human colon cancer (De Gottardi et al., 2004). In addition, mice deficient in FXR have increased colon tumorigenesis, increased intestinal permeability and WNT/β-catenin signaling, increased NFκb signaling, suggesting FXR as a tumor suppressor (Inagaki et al., 2006; Maran et al., 2009; Modica et al., 2008; Wang et al., 2008; Wolfe et al., 2011). It is suggested that the tumor suppressive role of FXR is two-fold. One, FXR regulates the synthesis and transport of bile acids preventing the accumulation of free bile acids and attenuating the tumorigenic effects of bile acids. And two, FXR suppresses colonic tumorigenesis independent of its regulation of bile acid levels, and directly regulates

transcription of anti-tumorigenic genes. Nevertheless, the suppression of FXR in intestinal epithelial cells clearly increases intestinal susceptibility to tumorigenesis. Therefore, reversing FXR silencing could be one mechanism exploited to slow the progression of colon cancer. The purpose of this study was to identify the molecular mechanisms involved in silencing FXR gene expression in human colon cancer so that it can be further determined whether FXR serves as a potential target for the treatment, prevention, and/or diagnosis of human colon cancer.

2.2: Overall Hypothesis:

Epigenetic mechanisms function to modify the transcription of genes without changing the underlying genomic sequence. Over the past decade, much research has been done elucidating chromatin modifications and cofactors involved in nuclear receptor induced transcription. However, due to recent technological advancements, this analysis has been extended to a more genome-wide scale. A primary focus is to further understand the epigenetic mechanisms involved in FXR-induced transcriptional regulation on a genome-wide scale and FXR silencing in human colon cancer. The *central hypothesis* is that epigenetic mechanisms, specifically interactions with orphan nuclear receptors, function to regulate binding of FXR in liver and intestine and that DNA methylation is the epigenetic mechanism responsible for FXR silencing in human colon cancer. These hypotheses will be tested via three Specific Aims. Following the completion of this study, a better understanding of FXR's tissue-specific functions on a genome-wide scale and of the molecular mechanisms involved in FXR silencing in human colon cancer is expected.

2.3: Specific Aims:

2.3.1: Specific Aim 1: Identify direct genome-wide FXR binding sites in liver and intestine.

In order to elucidate the epigenetic mechanisms involved in regulating genome-wide functions of FXR, a comprehensive understanding of direct FXR binding sites in liver and intestine, two main tissues for FXR function, is needed. In this aim, the following objectives were determined.

2.3.1a. Compare the degree of tissue-specific binding of FXR in mouse liver and intestine.

2.3.1b. Determine genome-wide binding distribution of FXR in liver and intestine.

2.3.1c. Motif analysis of FXR binding sites in liver and intestine.

2.3.1d. Pathway analysis of FXR binding in liver and intestine.

2.3.1e. Identification of novel FXR binding patterns to known and unknown target genes.

2.3.2: Specific Aim 2: HNF4α functions to co-regulate FXR function in the liver.

Genome-wide binding analysis of FXR in liver and intestine suggested the involvement of orphan nuclear receptors in regulating FXR function. HNF4α is a newly adopted orphan nuclear receptor shown to highly regulate many liver-specific functions. Therefore, the role of HNF4α in regulating FXR function on a genome-wide scale was investigated. In this aim, the following objectives were determined.

2.3.2a. Compare genome-wide binding of FXR and HNF4α in liver to assess the degree of overlapping binding.

2.3.2b. Determine distance of FXR and HNF4α binding from each other at shared target sites.

2.3.2c. Confirm binding of FXR and HNF4α to shared target genes and assess the dependence of binding of each factor on the presence of the other factor by chromatin immunoprecipitation (ChIP) followed by quantitative PCR (ChIP-qPCR) using FXR knockout mice.

2.3.2d. Determine whether FXR and HNF4α physically interact using co-immunoprecipitation (co-IP) assays.

2.3.2e. Determine the effect of HNF4α on regulating FXR-induced transcriptional activity of shared target sites.

2.3.3: Specific Aim 3: FXR is down-regulated in human colon cancer by DNA methylation of the FXR promoter.

As described, FXR is highly down-regulated in human colon cancer and mouse models of intestinal cancer. This aim was designed to elucidate the molecular mechanisms involved in silencing FXR in human colon cancer. In this aim, the following objectives were determined.

2.3.3a. Confirm degree of FXR and FXR target gene down-regulation in a mouse intestinal cancer model, APC^{min} mice, and in human colon cancer samples.

2.3.3b. Assess the effects of DNMT inhibition on FXR expression in human colon cancer cell lines.

2.3.3c. Identify any CpG islands located within the FXR promoter by methylated DNA immunoprecipitation (MeDIP) analysis.

2.3.3d. Correlate degree of FXR promoter methylation with FXR expression in human colon cancer cell lines by bisulfite sequencing.

2.3.3e. Assess the effects of siRNA knockdown of DNMTs on FXR expression in human colon cancer cell lines.

Chapter 3: Materials and Methods

3.1: Animals and Treatment:

All animals were housed in the AAALAC-accredited facilities at the University of Kansas Medical Center under a standard 12-hr light/12-hr dark cycle with access to chow and water *ad libitum*. All protocols and procedures were approved by the IACUC committee at the University of Kansas Medical Center.

3.1.1: Animals used for genome-wide binding studies and ChIP-qPCR.

Ten week-old wild-type (WT) or whole body FXR KO male mice (n=4 to 5 per group), in C57BL/6 genetic background, were fasted overnight and then gavaged with vehicle (1% methylcellulose, 1% Triton X-100 in PBS) or GW4064 (75 mg/kg). GW4064 was synthesized by the Chemical Discovery Laboratory at the University of Kansas, Lawrence, KS. Liver and intestines (ileum and colon) were collected 2, 4, or 8 hrs later.

For ChIP-qPCR analysis, optimal GW 4064 treatment groups for liver and intestine were determined to be 4 and 2 hr treatment times, respectively. Therefore, these tissues were used for FXR and/or HNF4α ChIP-qPCR analysis.

3.1.2: Animals for co-IP assays.

For co-IP assays, 4 month-old WT and FXR KO mice (n=3 per group), in C57BL/6 genetic background, were fed control diet or a diet supplemented with 1% (w:w) cholic acid (CA). Liver tissues were isolated after 5 days of treatment and whole cell lysates were prepared for immunoprecipitation assays.

3.1.3: Animals for FXR expression.

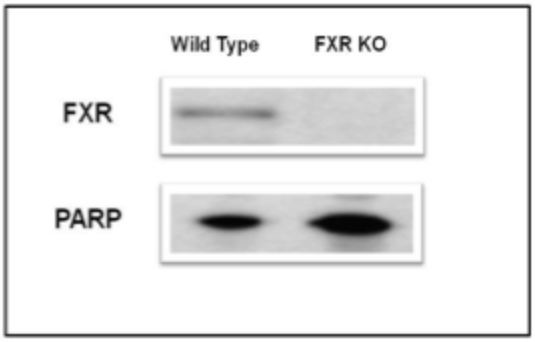
WT and APC^{min} mice in C57BL/6 genetic background were obtained from Jackson Laboratories (Bar Harbor, Maine). One-year old male and female APC^{min} mice (n=4 per group) were used to evaluate the expression of FXR. APC^{min} mice carry a nonsense mutation in the APC (adenomatous polyposis coli) gene, are a well known intestinal carcinogenesis mouse model, and develop tumors in the small intestine (Su et al., 1992).

3.2: ChIP-sequencing:

3.2.1: Chromatin immunoprecipitation for sequencing.

This procedure was performed by Genpathway, Inc (San Diego, CA). Briefly, flash frozen tissues were fixed in formaldehyde before quenching with glycine. The nuclei were extracted and sonicated to yield 500-1000 bp DNA fragments. Chromatin was pre-cleared with blocked Staph A cells (Pansorbin, CalBiochem, San Diego, CA) before incubation with a ChIP-quality anti-FXR antibody (H-130x, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibody specificity for mouse FXR was validated and is presented in **Figures 5a** and **5b**. Samples were incubated with prepared Staph A cells to extract antibody-chromatin complexes, followed by washing and elution. DNA fragments associated with the FXR antibody were released and purified. The purified DNA fragments were first analyzed by quantitative PCR (qPCR) with primers amplifying known FXRREs of target genes. The primers are presented in **Table 1a.**

a.


Wild Type
FXR KO
FXR
PARP

b.

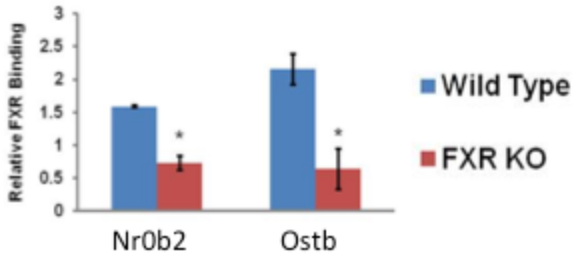

Relative FXR Binding
3
2.5
2
1.5
1
0.5
0
*
*
Nr0b2
Ostb
Wild Type
FXR KO

Figure 5:

Figure 5: FXR antibody specificity. a) Western blot analysis of nuclear extracts from WT (lane 1) and FXR KO (lane 2) mouse liver using antibodies against FXR or loading control protein, PARP. b) ChIP-qPCR analysis of WT and FXR KO mouse livers. Fixed, sonicated chromatin prepared from WT and FXR KO mouse livers was immunoprecipitated using antibody against FXR. The IP DNA was then PCR amplified using primers specific for known FXR binding sites in Shp (*Nr0b2*) and Ostβ (*Ostb*) genes.

For HNF4α ChIP-Seq, ChIP experiments were done as previously reported (Schmidt et al., 2010). Raw data were obtained from ARRAYEXPRESS Archive (European Bioinformatics Institute; E-TABM-722; Schmidt et al., 2010).

3.2.2: Sequencing analysis of ChIP DNA.

ChIP DNA fragments associated with the FXR antibody were amplified using the Illumina ChIP-Seq DNA Sample Prep Kit (San Diego, CA). The DNA libraries generated were further tested by qPCR at the same specific genomic regions as the original ChIP DNA to assess quality of the amplification reactions. DNA libraries were sequenced on a Genome Analyzer II by Illumina Sequencing Services. After sequencing, data files were processed through Genome Analyzer Pipeline Software (Illumina) and mapped to the genome. Enriched intervals, referred to as peak values, were defined when a given region appeared more than 20 times (a conservative threshold was arbitrarily set at > 20). A genomic region containing more than one enriched interval overlapping by at least one base pair was defined as an active region. The wiggle (WIG) formatted files are stored in the UCSC database (http://genome.ucsc.edu/goldenPath/customTracks/custTracks.html#Mouse) and can be downloaded using UCSC Genome Browser (Kent et al., 2002).

The distribution of FXR binding sites relative to target gene TSS was analyzed by JMP 7.0. Analysis was done to determine the average peak value of FXR binding as well as to determine the total number of FXR binding events in relation to distance of site from a gene TSS.

Histograms of FXR and/or HNF4α binding to target genes were generated using Affymetrix Integrated Genome Browser (IGB) or UCSC Genome Browser (Kent et al., 2002; Nicol et al., 2009).

3.2.3: ChIP-Seq analysis of FXR and HNF4α in mouse livers.

ChIP-seq for FXR was performed on mouse livers as described above, and for HNF4α as previously described (Schmidt et al., 2010). Raw FXR and HNF4α ChIP-sequencing data were generated from single end sequencing on an Illumina Genome Analyzer and were re-analyzed using Model-based Analysis of ChIP-Seq (MACS). It is important to note that ChIP-seq analysis was done in livers from different mice. Total FXR binding sites in liver were compared to total HNF4α binding sites in liver. The binding frequency of HNF4α relative to FXR binding site was analyzed using BEDtools (Quinlan and Hall, 2010).

3.2.4: Motif identification.

ChIP-seq peaks unique to liver, intestine or both were used to define tissue-specific FXR-DNA binding motifs. Using the midpoint from the 500 highest peak values for each tissue as a reference, 48 bp sequences flanking either side were retrieved from the UCSC Browser (Kent et al., 2002). Each file was then independently run using MEME (Multiple Em for Motif Elicitation; Bailey and Elkan, 1994). MEME uses a position-dependent letter probability matrix that describes the probability of each possible letter at each position in the pattern. Individual MEME motifs do not contain gaps. Patterns with variable-length gaps were split by MEME into two or more separate

motifs. Motifs containing or resembling the canonical ‘AGGTCA’ motif (which were also the highest scoring motifs) were cross compared between tissues.

3.2.5: Pathway analysis.

Peaks identified in ChIP-seq data, in both liver and intestine, that were located 0-2 kb upstream from TSSs were analyzed using the Functional Annotation Tool in DAVID (DAVID; http://www.david.niaid.nih.gov; Dennis et al., 2003). For a pathway or process to be defined, the threshold count was set at 2 with a minimum EASE (Expression Analysis Systematic Explorer) score, a modified Fisher Exact Test, of 0.1. Only Bonferroni corrected p-values with false discovery rates (FDRs) less than or equal to 0.1 were accepted.

Pathway analysis of direct target genes shared by FXR and HNF4α in mouse liver was also analyzed by DAVID as described above. Processes for this analysis with a p-value less than or equal to 0.05 were accepted.

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3.3: ChIP-qPCR Assays.

ChIP assays were performed on livers and intestines of mice treated with GW4064 or vehicle once for 4 and 2 hrs, respectively. Briefly, flash-frozen livers were minced and fixed in 1% formaldehyde for 15 min and then quenched with 0.125 M glycine. Nuclear extracts were prepared and sonicated to yield 500-1000 bp DNA fragments. Sonicated chromatin was aliquoted to yield 20 mg tissue equivalents for each immunoprecipitation assay.

For FXR ChIP-qPCR, samples were pre-cleared with protein agarose G-salmon sperm DNA beads (Millipore Corp, Billerica, MA) before incubation with an IgG antibody or the anti-FXR antibody (H-130x, Santa Cruz Biotechnology, Inc). Samples were incubated with prepared protein agarose G-salmon sperm DNA beads in order to extract antibody-chromatin complexes. Complexes were washed and eluted with immunoprecipitation elution buffer. DNA fragments associated with the FXR antibody were released by incubating samples in a 450 mM NaCl solution at 65^{o}C for 5 hrs. RNA and protein were degraded by treating chromatin with RNase A and proteinase K. DNA fragments were purified by standard DNA column purification. The purified DNA fragments that were bound by FXR were analyzed by qPCR using primers that amplify FXR binding sites (**Table 1a-1b**). QPCR reactions were carried out using MaximaTM SYBR Green (Fermentas; Glen Burnie, MD). Data was analyzed as fold change over IgG negative controls.

For HNF4α ChIP-qPCR analysis, assays were done as described above. Livers of WT and FXR KO mice treated for 4 hrs with vehicle or GW4064 were used for analysis. Immunoprecipitation was done using anti-HNF4α antibody (PP-H1415-00, R&D Biosystems, Minneapolis, MN) and Dynabeads Protein G (Invitrogen, Carlsbad, CA). The purified DNA fragments were analyzed by qPCR with primers amplifying shared FXR and HN4α binding sites (**Table 1a** and **1c**).

3.4: Co-IP:

Co-IP assays were done on whole cell liver extracts from WT mice fed diet with or without 1% CA and FXR KO mice fed control diet. The assay was done using a co-IP kit from Invitrogen. Specifically, whole cell lysates from mouse livers were prepared

Table 1a: Primers for ChIP-qPCR assays.

Primer Name	Primer Sequence: 5'-3'
Nr0b2 -320 to -220 For (for initial q-PCR)	CTGGTTGAGCGCCTGAGAC
Nr0b2 -320 to -220 Rev (for initial q-PCR)	CTGCCTGGATGCCCTTTATC
Ostb -220 to -150 For (for initial q-PCR)	CCGCAATGGCAGATCATAC
Ostb -220 to -150 Rev (for initial q-PCR)	GTGAATGACCCCACGAATG
Osta -1245 to -1145 For (for initial q-PCR)	CAGTGGAAGTGGCTTGAGTC
Osta -1245 to -1145 Rev (for initial q-PCR)	GGGCAGGAGAGGAAGCTAAG
Untr6 For	TCAGGCATGAACCACCATAC
Untr6 Rev	AACATCCACACGTCCAGTGA
Shp -320 to -220 For	GCCTGAGACCTTGGTGCCCTG
Shp -320 to -220 Rev	CTGCCCACTGCCTGGATGC
Shp +950 to +1050 For	CAGTCCACGCCCTCAGCCC
Shp +950 to +1050 Rev	GGCAGGAGGAGGTCTGAAAGC
Ostb -220 to -150 For	TGGGCTCCTGGCACTTTCGG
Ostb -220 to -150 Rev	TGGGACTTCAGGCTGGGTGG
Osta -1245 to -1145 For	CAGCTCCCTCTTGCCCTCC
Osta -1245 to -1145 Rev	TAGACAGTTCACCATGTCTCTTGAGTCC
Bsep -100 to -10 For	CCTCTCACCAGGCTCTCTACC
Bsep -100 to -10 Rev	CGCCACTGTGGAAAGTCAGGG
Fgf15 1900 to 2000 For	CCTGCCTGGTGGCTCTGTCTC
Fgf15 1900 to 2000 Rev	GGATAATCCGCAACTCCTCCCGCC
Slc9a8 (Nhe8) -5350 to -5250 For	AGAAGAGCCTAGGACTTTCCCACA
Slc9a8 (Nhe8) -5350 to -5250 Rev	TCTGGCTGGTCACACTGGTTAAGA
Slc25a3 -7770 to -7670 For	ATTGGGTGTAGCTTGAGTGGAGGA
Slc25a3 -7770 to -7670 Rev	TGACCCATAAAGGGTGTGGTGGTT
Hmga1 -3650 to -3550 For	TGGTTTGGGCTAGGTCAGGTTGAT
Hmga1 -3650 to -3550 Rev	CCCAAATGTGAATGTCCCGCAGTT
Pddc1 -470 to -370 For	TCTGGATCTTCACAAACCGGCAGA
Pddc1 -470 to -370 Rev	TCCCTGAGTGGTCGTTTCCTGATT
Fcna -950 to -850 For	CTGGAGGGACAGAGGGAGGTCAGT
Fcna -950 to -850 Rev	GGCCAGACTAGGCCAGCTAT
Fcna -4570 to -4470 For	TTGTCCAGCCCAAGGACATCAAGA
Fcna -4570 to -4470 Rev	TTACAACGATGCCTTCACCCTCAC
Sumo3 -7370 to -7270 For	TGCCTTTCCTTCAGTAAGCAGCCA
Sumo3 -7370 to -7270 Rev	AAACCAGGAAGGCTGTGAGGACA
Acads 18560 to 18660 For	AGCTCTCTGGACACAACAGACACA
Caprin1 270 to 370 For	CGGTCACTCCAAGTGCCCTTCT
Caprin1 270 to 370 Rev	CAAGTGGCACCCGTTGCCTT
Actg1 -5260 to -5160 For	TTTCCTGGTCCCTCTTGGCCTTTA

Table 1b: Primers for ChIP-qPCR assays.

Primer Name	Primer Sequence: 5'-3'
Actg1 -5260 to -5160 Rev	GGAGCAGGAGAACGCTGGG
Vkorc1 870 to 970 For	TGAGCCATCTCACCAACTCCTGAA
Vkorc1 870 to 970 Rev	ACCTGGTGGAAGTGAAAGAAGCAG
Tcfap2e 3080 to 3180 For	GACCCAGTCGAAGGGAAAGTTG
Tcfap2e 3080 to 3180 Rev	TCTGCCTTGGGATACTGGAGTG
Cks2 -100 to -10 For	TACTCCCGCCCTCGCAATCTTG
Cks2 -100 to -10 Rev	CCAACGACCGCAGGCCCAAC
Rorc (Rorg) 10650 to 10750 For	AGAGACAGCTCCCCAATTAAGAGTG
Rorc (Rorg) 10650 to 10750 Rev	GTCTTGGATGTCCCGCTGTTGTG
Slc10a1 (Ntcp) -8100 to -8000 For	GCTGTGTTTACCCTCTGCCATC
Slc10a1 (Ntcp) -8100 to -8000 Rev	CCCTAACGTGCCTTGACCC
Fga -5680 to -5580 For	TGGGTCACAGTTGTGTCTCTCATAGC
Fga -5680 to -5580 Rev	GTGGAATCTGCCCTGCCATGTTTA
Fga -190 to -290 For	GATGTTGCCTTTGCCCTGGTCTTT
Fga -190 to -290 Rev	CTGTGTGTGGGATTCAGAGTTCATC
Mir499 -2400 to -2300 For	CTCTCCTGCACTTTGGCAACATGA
Mir499 -2400 to -2300 Rev	AAGTTTCCAGGGTAAAGGCCAGGT
Mir126 +8650 to +8750 For	GGCTGCACAAATTCAGGTCAGCAA
Mir126 +8650 to +8750 Rev	AGGATGGACTACTGGAACCCAACA
Mir126 +9450 to +9550 For	ATACCTGCCCAAGGCAGTTGAGAA
Mir126 +9450 to +9550 Rev	CACCCATCAAACTCAAGGCCAGAA
Bccip -380 to -280 For	ACAACCTGCACGCCGAGATAA
Bccip -380 to -280 Rev	TCTGTGTGTAGTGGTTTGGCCCAT
Nat2 -580 to -480 For	TACTCAACAGTGTGGTGCCAGC
Nat2 -580 to -480 Rev	CATGATATCAGGGTCCATGGGTCA
Rarres2 -150 to -100 For	TGGTGCTCCAGGCCCTCC
Rarres2 -150 to -100 Rev	CCCTCTCACCCTCTCATCTCCC
Il17b -8280 to -8180 For	CCATGTATAACACAAGTCAAGCTGTCTCC
Il17b -8280 to -8180 Rev	CTTCCCTAGTCTTTGCCCTGGTTT
Smurf1 18350 to 18450 For	TGCCCACTGCCCACATAAGTTAGA
Smurf1 18350 to 18450 Rev	ACTGCTCATTGGCTCCCTACAACA
Gpbp1 -210 to -110 For	TACTTGGGCCAGAAGGTTCTGTGGT
Gpbp1 -210 to -110 Rev	AGGTTGAGAAGTAAGAGGAGGGAG
Pcx -60 to +40 For	GCAGTCTAGTGCTGGAGAACTTTG
Pcx -60 to +40 Rev	ATTACCTTGTACCAGCCAAGCACC
Cnnm3 -320 to -220 For	CGCAAGCGCAAACCCACATACAAA
Apoc3 -50 to 50 For	TCAGGCTCTGGTCTGGACTGCTCA
Apoc3 -50 to 50 Rev	TTATATTGGCTCCAGGATGGGACAGC
Baat -150 to -50 For	CACTAGAAGCCCGATGCTTTCA

Table 1c: Primers for ChIP-qPCR assays.

Primer Name	Primer Sequence: 5'-3'
Baat -150 to -50 Rev	AGTAGGCTGAACCCAGAGAAGAGA
Apoe -2100 to -2000 For	TGAGGTGGTAGCTTGTGCTGACTT
Apoe -2100 to -2000 Rev	TGCTGAACTTCCAGGAACTCCGTT
Sqsm1 13000 to 13100 For	CACTGCACATGTGTGTTTCTGTGT
Sqsm1 13000 to 13100 Rev	AGGGTGTGGACAGTGTTGAAGACA

according to protocol, and then immunoprecipitated using magnetic beads covalently linked with an antibody against FXR (H-130x, Santa Cruz Biotechnology, Inc). Immunoprecipitates were then pooled and analyzed by western blot for the detection of HNF4α (PP-H1415-00, R&D Biosystems).

3.5: Cell Culture:

CHO cells were used for luciferase expression assays. These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Omega Scientific, Tarzana, CA), 1% penicillin/streptomycin, and L-proline (50 μg/mL). Caco-2, HT-29, and SW620 cells were used for analyzing FXR expression by real-time PCR, FXR promoter CpG island methylation, and siRNA knockdown experiments for DNA methylation studies. Caco-2 and HT-29 were cultured in DMEM and SW620 cells were cultured in Leibovitz's L-15 media, both supplemented with 10% FBS and 1% penicillin/streptomycin. Caco-2 and HT-29 cells were incubated in a humidified 37^{o}C incubator with 5% CO_2, whereas SW-620 cells were incubated in a humidified 37^{o}C incubator with 0% CO_2.

3.5.1: Plasmid constructs, transfection and luciferase expression assays.

Fragments containing an FXRRE (IR-1), in the promoter and downstream regulatory regions of the *Nr0b2* gene were amplified and cloned upstream of the luciferase gene into firefly luciferase vector pGL4.23[luc2/minP] (Promega, Madison, WI) as previously reported (Li et al., 2010). An active HNF4α binding site upstream of *Baat* gene TSS has already been previously reported (Inoue et al., 2004) and was used as positive control for HNF4α transcriptional activity. For this study, this region was amplified from mouse genomic DNA by PCR using pairs of primers containing XhoI and HindIII restriction enzyme sites, and cloned into the pGL4.23 firefly luciferase vector. The sequences of the primers are: forward 5'-CACAACTCGAGAATGGCTAAGACTATAGAT-3', and reverse 5'-CTGAGGAAGCTTTCTTAGTATTTCCCTCCTC-3'. The sequences of these constructs were confirmed by DNA sequencing.

CHO cells were grown to 90% confluence in 12-well plates, and were transiently transfected with the various reporter gene constructs as well as pCMV-ICIS human FXR and/or pCMV-sport6 mouse HNF4α (Open Biosystems, Huntsville, AL), pSG5 human RXRα, and phRGTK-renilla luciferase vector (Promega, Madison, WI). Transient transfection was done using Turbofect (Fermentas, Inc., Glen Burnie, MD) according to the manufacturer's instructions. Twenty-four hrs after transfection, cells were trypsinized and plated into 96-well plates and treated with 100 nM GW4064, 0.1% DMSO (control), or no treatment for HNF4α-alone groups. Twenty-four hours later, firefly luciferase and renilla luciferase activities were quantified using a Dual-Glo Luciferase Kit from Promega (Madison, WI) with a Synergy-HT plate reader (Bio-Tek Instruments, Inc., Winooski, VT). FXR/RXR expression vectors were co-transfected with increasing

amounts (3, 10, and 30 ng) of HNF4α expression vector with the addition of FXR synthetic ligand, GW4064. The Shp promoter and downstream IR-1s cloned into luciferase expression vectors were used to assess the effects of HNF4α on transcriptional activity of FXR. The transcriptional activity of increasing amounts of HNF4α expression vector (10, 50, and 100 ng) on the Shp promoter and downstream regulatory region, as well as a positive control gene, Baat, was also measured by luciferase assay. The firefly luciferase activity value was normalized as a ratio over renilla luciferase and expressed as firefly luciferase activity/renilla. The data are presented as the average of six wells and the experiments were repeated at least twice.

3.5.2: Colon cancer cell lines and FXR expression.

For analysis of FXR expression, HT-29, Caco-2, and SW620 cells were grown for 3, 7, 14, and 21 days as previously reported (De Gottardi et al., 2004). RNA and DNA were then extracted as described below. The purified genomic DNA from these cells was used for bisulfite analysis and MeDIP, and RNA was prepared for real-time qPCR.

3.5.3: Azacytidine treatment.

As an initial screen, cDNAs prepared from 11 different colon cancer cell lines treated with a clinically used DNMT inhibitor, azacytidine (AZA, Sigma, St. Louis, MO), were used to measure mRNA levels of FXR by real-time qPCR. Complimentary DNAs were obtained from Dr. Vadevil Ganapathy from Georgia Health Sciences University. To confirm these preliminary results, colon cancer cell lines, HT-29 and SW620, were treated with AZA and mRNA levels of FXR were measured by real-time qPCR.

Complimentary DNA from these samples was prepared from 1 million cells were plated in 10-cm plate treated with or without 2 μg/ml azacytidine for three days. COL1A2 and FXR mRNA levels were determined by real-time qPCR. The COL1A2 gene encodes for the collagen-a2 protein and has been shown to be methylated in colon cancer cell line SW620 (Sengupta et al., 2003). Therefore, the expression of this gene in response to AZA treatment was used as a positive control.

3.6: Human Colon Cancer Samples and FXR Expression.

Two sets of human colon specimens were used to analyze mRNA levels of FXR and FXR target genes by real-time qPCR. One set was cDNA prepared from normal and cancerous colon tissues obtained from OriGene Technologies (Rockville, MD). The patient sample pathology reports can be obtained from OriGene's website (http://www.origene.com; HCRT501). The second sample set was from the Biospecimen Core of the Cancer Center in the University of Kansas Medical Center. The patient sample pathology report for this set is listed in **Table 2**. In addition, cDNA prepared from human colon cancer cells lines HT-29, NCM460, KM12L4, Colo201, SW620, and LS-174T were obtained from Dr. Vadevil Ganapathy from Georgia Health Sciences University. Briefly, cells 1 million cells were plated in 10-cm plates in normal growth media, grown for 3 days, and RNA was extracted. RNA extraction and real-time qPCR protocols for human colon specimens are described below. All prepared cDNA was used to measure mRNA levels of FXR, OSTα, and OSTβ. Messenger RNA levels were normalized to β-actin or GAPDH.

3.7: MeDIP Assay:

The MeDIP assay was preformed as previously published (Weber et al., 2005). Specifically, HT-29 and SW620 cells were plated at 1 million cells in 10-cm plate and grown for 3 days in regular cell culture growth medium. Genomic DNA was extracted and purified as described below. Around 4 μg of sheared DNA was diluted into 450 μl of TE buffer, denatured in boiling water for 10 mins and immediately cooled on ice for 10 mins. Next, 50 μl of 10x immunoprecipitation (IP) buffer (100 mM Na-Phosphate pH 7.0, 1.4 M NaCl, 0.5% Triton X-100) and 50 ng of the 5-methylated-cytosines (5mC) antibody (MAb-335MEC-100, Diagenode Inc., Sparta, NJ) were added to the DNA solution and samples were incubated overnight at 4^{o}C with overhead shaking. Magnetic Dynabeads (Invitrogen, M-280 sheep anti-mouse IgG) were pre-washed with PBS + 0.1% BSA for 5 mins at room temperature with shaking. Beads were collected with a magnetic rack and resuspended with 40 μl of 1x IP buffer. DNA samples with anti-5mC antibody were added to the beads and incubated with overhead shaking at 4^{o}C for 5 hrs. Beads were washed two times with 1x IP buffer for 10 mins at room temperature with shaking. Beads were resuspended in 250 μl proteinase K digestion buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS) and 7 μl of proteinase K (10mg/ml) and incubated at 50^{o}C for 3 hours with shaking. Immunoprecipitated DNA was phenol/chloroform purified and dissolved in TE buffer.

Precipitated DNA was analyzed by qPCR using primers designed to amplify positive control methylated CpG islands within the collagen-a2 promoter (Sengupta et al., 2003) and FXR promoter CpG island by SYBR green chemistry. Primers used to amplify these CpG island are: FXR CpG forward 5'-

Table 2: KUMC cancer center biospecimen pathology reports summary.

Patient #	gender	race	age	tumor site	diagnosis	Histolograde	Invasion	config.	ploidy	stage	tumor type	tumor border	tnm	pre-existing polyp
1119	Male	caucasian	80	cecum	malignant colon ca	well differentiated	submucosa	exophytic (polypoid)	aneuploid	I	adenocarcinoma, adenoma	infiltrating	pT1pN0MX	
1546	Male	african-american	57	sigmoid colon	malignant colon ca	well differentiated	muscularis propria	exophytic (polypoid)		I	adenocarcinoma	infiltrating	pT2pN0	yes
1296	Female	asian or pacific	80	left colon	colon ca malignant	moderately differentiated moderately	muscularis propria muscularis	exophytic (polypoid); infiltraitve exophytic	aneuploid	II A	adenocarcinoma	infiltrating	pT3pN0	yes
1545	Female	caucasian	86	cecum	colon ca malignant	differentiated	propria	(polypoid)	aneuploid	II A	adenocarcinoma	infiltrating infiltrating; vascular;	pT3bpN0	no
1337	Female	caucasian	70	cecum	colon ca malignant	moderately differentiated moderately	muscularis propria	infiltraitve, ulcerating exophytic (polypoid)	aneuploid	III B	adenocarcinoma	perineural	pT3dpN1	no
1450	Female	caucasian	69	sigmoid	colon ca malignant	differentiated moderately	muscularis propria	aneuploid	III B	adenocarcinoma	infiltrating	pT3apN1	no	
1474	Male	caucasian	65	sigmoid colon	colon ca malignant	differentiated moderately	propria	ulcerating exophytic	aneuploid	III B	adenocarcinoma, adenoma	infiltrating; perineural	pT3cpN1	yes
1531	Female	caucasian	60	sigmoid	colon ca	differentiated		(polypoid)	aneuploid	III B	adenocarcinoma, adenoma	infiltrating	pT3pN1	yes
1573	Female	caucasian	48	sigmoid, rt lobe of liver	malignant colon ca	moderately differentiated	muscularis propria	polyploid		III B	adenocarcinoma, adenoma	pushing, perineural	pT3cpN1M1	yes
1353	Male	caucasian	24	leocecal valve, cecum	malignant colon ca	well differentiated	muscularis propria	exophytic (polypoid)		III C	adenocarcinoma	infiltrating	pT2pN2	no
1340	Female	caucasian	44	sigmoid colon, liver omenitum, sm intest., ejunum, rectum, bladder, peritoneal	malignant colon ca recurrent					IV	adenocarcinoma		TXNXM1	
1415	Male	caucasian	74		malignant colon ca	moderately differentiated			aneuploid	IV	adenocarcinoma			yes

GTTTGAGACAAGCCTGGGCAACAT-3', and FXR CpG reverse 5'-ATTTCGGGTTCAAGCGGTTCTCCT-3'; COL1A2 CpG forward 5'-TGCAGACAACGAGTCAGAGTTTCC-3', and COL1A2 CpG reverse 5'-GGGCTGGCTTCTTAAATTGGTTCC-3'. Primers designed to amplify a non-methylated housekeeping gene (UBE2B) were used as a negative control and sequences are as previously reported (Sorensen et al., 2010).

3.8: Bisulfite Sequencing:

Genomic DNA was isolated from human colon cancer cell lines, Caco-2, HT-29, and SW620, by standard DNA extraction protocols. Bisulfite conversion of genomic DNA was performed using EZ DNA Methylation Kit (Zymo Research Corporation, Irvine, CA).

Six predicted CpG islands were identified in the FXR gene determined by MethPrimer (http:// www.urogene.org/methprimer/; Li and Dahiya, 2002). **Figure 6a** shows the relative locations of these predicted CpG islands as well as the locations of each CpG island from the FXR gene TSS. The CpG islands found upstream of tumor suppressor gene TSS are considered the most common site of methylation for gene silencing (Baylin et al., 1998). Therefore, only the CpG island located upstream of FXR TSS was investigated for methylation. Primers specific for bisulfite converted DNA were used to amplify this CpG island located upstream of FXR TSS. The bisulfite specific primers used to amplify converted FXR promoter CpG island were: forward 5'-AGTGAGAGAGATATGAAATATGTTT -3'; and reverse 5'-

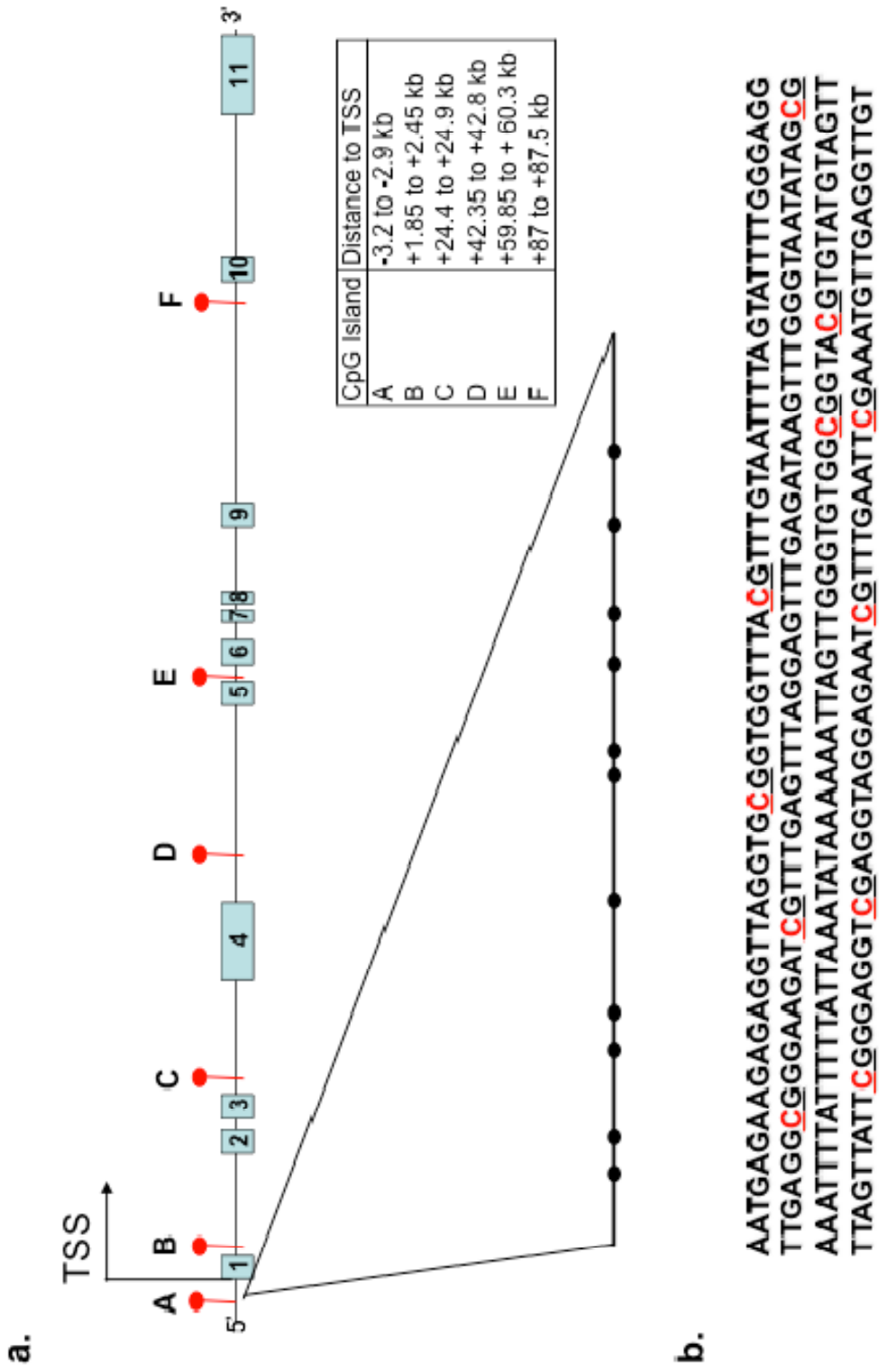
a.
TSS
A
B
C
D
E
F
5'
3'
1
2
3
4
5
6
7
8
9
10
11
CpG Island | Distance to TSS
A | -3.2 to -2.9 kb
B | +1.85 to +2.45 kb
C | +24.4 to +24.9 kb
D | +42.35 to +42.8 kb
E | +59.85 to + 60.3 kb
F | +87 to +87.5 kb
b.
AATGAGAAGAGAGGTTAGGTGCGGTGGTTTACGTTTGTAATTTTAGTATTTTGGGAGG
TTGAGGCGGGAAGATCGTTTGAGTTTAGGAGTTTGAGATAAGTTTGGGTAATATAGCG
AAATTTTATTTTTATTAAATATAAAAATTAGTTGGGTGTGGCGGTACGTGTATGTAGTT
TTAGTTATTCGGGAGGTCGAGGTAGGAGAATCGTTTGAATTCGAAATGTTGAGGTTGT

Figure 6:

Figure 6: Predicted CpG islands within the FXR (*NR1H4*) gene. a) There are 6 predicted CpG islands within the FXR gene as determined by MethPrimer CpG island predictor software. CpG island A is located approximately -3.2 to -2.9 Kb upstream of the TSS and CpG island B is located around 1.85 to 2.45 Kb downstream of the TSS, within the first intron of the FXR gene. CpG islands C, D, E, and F are located at approximately 24.4 to 24.9, 42.35 to 42.85, 59.85 to 60.35, and 87 to 87.5 Kb downstream of the TSS, within the 3^{rd}, 4^{th}, 5^{th}, and 9^{th} introns of the FXR gene. CpG island A is shown as a dashed line and CpG sites illustrated as black circles. b) The predicted sequence of FXR promoter CpG island (CpG island A) after bisulfite sequencing assuming complete CpG methylation. There are 11 predicted CpG sites within this CpG island. The dinucleotide CG is underlined throughout the sequence.

CTATCACCTAAACTAAAAAACAATAA -3'. After PCR amplification, fragments were gel purified and cloned into a TOPO TA vector (Invitrogen, Carlsbad, CA). The bisulfite converted CpG island clones were then purified and sequenced using ACGT, Inc. (Wheeling, IL) sequencing services. Bisulfite sequencing will convert non-methylated cytosine to uracil, but will be unable to convert 5-methylated cytosine. Sequencing of bisulfite converted DNA reveals the presence of methylated CpG sites within this CpG island. There are 11 predicted CpG sites within the FXR promoter CpG island. **Figure 6b** shows the predicted sequence of this CpG island after bisulfite conversion. Bisulfite sequencing was done on genomic DNA extracted from Caco-2, HT-29, and SW620 that were grown for 3, 7, 14 and 21 days. Data are reported as percent methylation of the CpG island found within the FXR gene promoter, or number of confirmed CpG sites out of 11 predicted CpG sites.

3.9: SiRNA Knockdown:

DNMT 1 and 3B are enzymes commonly associated with aberrant DNA methylation (Jones and Baylin, 2002; Rhee et al., 2002). Therefore, to assess whether DNMT 1 and/or 3B are responsible for FXR gene methylation in SW620 resulting in FXR down-regulation, siRNAs designed to knockdown expression of DNMT1 or DNMT3B were used. The smartpool siRNAs for knockdown of these two DNMTs and non-targeting siRNA were obtained from Dharmacon (Lafayette, CO). SW620 cells were plated at 30% confluence and reverse-transfected with DMNT 1, DNMT 3B, or non-targeting siRNAs using Turbofect (Fermentas, Inc.) for 96 hrs. DMNT 1 and 3B knockdown was confirmed by real-time PCR analysis before determining endpoints.

After knockdown of DNMT 1 and/or 3B, total RNA were prepared. RNA was used to quantify mRNA levels of FXR and positive control gene for DNMT inhibition, COL1A2.

3.10: RNA Extraction and Real-Time qPCR:

Total RNA was isolated from mouse ileum and colon, human colon samples, and colon cancer cells lines using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Complimentary DNA was prepared by standard RT-PCR methods using random primers (Fermentas, Inc.). Mouse tissues were used to measure FXR mRNA in ileum and colon and cDNA prepared from human colon samples and cell lines were used to measure mRNA levels of FXR, SHP, OSTα, and/or OSTβ. Complimentary DNA prepared from SW620 cells used in DNMT siRNA knockdown experiments were used to measure mRNA levels of DNMT 1, DNMT 3B, FXR, and the positive control gene, COL1A2. All real-time qPCR reactions were done using standard SYBR green chemistry and an ABI Prism 7900 Detection system (Applied Biosystems, Foster City, CA). The mRNA levels of these genes were normalized to 18s mRNA levels for the mouse samples and β-actin or GAPDH for the human samples. The primer sequences used for real-time qPCR are listed in **Table 3**.

3.11: Genomic DNA Extraction for MeDIP and Bisulfite Sequencing:

Genomic DNA was extracted from human colon cancer cell lines Caco-2, HT-29, and SW620 for MeDIP and bisulfite sequencing as previously described (Sengupta et al., 2003; Weber et al., 2005). Specifically, cancer cells were harvested then resuspended in DNA lysis buffer (10mM Tris (pH 7.5), 10mM EDTA, 10mM NaCl , 0.5%

Table 3: Real-time qPCR primers.

Primer Name	Forward	Reverse
Mouse		
FXRα	CTTGATGTGCTACAAAAGCTGTG	ACTCTCCAAGACATCAGCATCTC
FXRβ	CATACAAGGGCTAATGAAGTTTACCA	TTTTGACGCCTTCTGTAATGC
18S	CCGAAGCGTTTACTTTGAAAAAA	TTCATTATTCCTAGCTGCGGTATC
Human		
FXR	TGCATTGAAGTTGCTCTCAGGT	CGCCTGACTGAATTACGGACA
SHP	AGCTGGAAGTGAGAGCAGATCC	AGAAGTGCGTAGAGAATGGCG
Ostα	CTACACCTGGGTGAGCAGAA	AGAGGAATAGGGAGGCGAAC
Ostβ	GCAGCTGTGGTGGTCATTAT	TAGGCTGTTGTGATCCTTGG
DNMT 1	TGTACCGAGTTGGTGATGGTGTGT	TGCTGCCTTTGATGTAGTCGGAGT
DNMT 3B	ATTGTTTGATGGCATCGCGACAGG	ACAGCAATGGACTCCTCACACACT
GAPDH	GGTGGTCTCCTCTGACTTCAA	GTTGCTGTAGCCAAATTCGTTGT

sodium dodecyl sulfate) and 1mg/ml Proteinase K and incubated at 55^{O}C for 5 hours or overnight. Ethanol was added to precipitate DNA, DNA was collected, washed with 75% ethanol, and redissolved into DNase free water. Genomic DNA was further purified by standard phenol/chloroform extraction methods. Purified DNA was briefly sonicated to fragment the genomic DNA, and then column purified using standard PCR purification kits (Fermentas). Purified, fragmented genomic DNA was then used for MeDIP and bisulfite sequencing analysis.

3.12: Western Blot:

For western blot analysis of co-IP samples, 15 μg of whole cell input protein lysate and total FXR IP fractions (40 μl) were loaded onto a 12% acrylamide/Tris-HCL gel and electorphoresed 45 mins at 190 millivolts (mV). Protein was then transferred to 0.45 μm PVDF membrane for 60 mins at 100 mV. Blots were labeled with antibody

against HNF4α (PP-H1415-00, R&D Biosystems) overnight and then detected by standard chemiluminescent detection methods (ECL, Amerisham/ GE Healthcare Biosciences, Pittsburg, PA).

3.13: Statistical Analysis:

Statistical analysis was done using Student's t-test and p-value < 0.05 was considered statistically significant. The groups compared are listed as the following. For ChIP-qPCR analysis, agonist treated or KO animals were compared to vehicle treated WT controls. For HNF4α-alone luciferase assays, CHO cells transfected with luciferase vector containing response elements were compared to luciferase vector control. For luciferase assays with HNF4α and FXR, GW4064 treated CHO cells with FXR-RXR alone were compared to DMSO treated control cells and cells with increasing amounts of HNF4α vector with GW4064 treatment were compared to CHO cells with FXR-RXR and GW4064. For animal studies, ileum and colon from APC^{min} mice were compared to those from WT control mice. Human colon cancer samples from stages I, II, III, and IV were compared to normal human colon samples. For AZA treated colon cancer cell lines, cells treated with AZA were compared to vehicle treated cells. For MeDIP analysis, PCR reactions done to amplify CpG islands were compared to a negative control region, as well as MeDIP from SW620 cells compared to that of HT-29 cells. For SiRNA knockdown experiments, SW620 cells transfected with DNMT 1 or 3b siRNAs were compared to non-targeting siRNA control cells.

Chapter 4: Genome-wide Tissue Specific FXR Binding in Mouse Liver and Intestine

(*Hepatology* 51(4):1410-1419)

4.1: Abstract:

FXR is a bile acid-activated TF belonging to the nuclear receptor superfamily. FXR is highly expressed in liver and intestine, and cross-talk mediated by FXR in these two organs is critical in maintaining bile acid homeostasis. FXR deficiency has been implicated in many liver and intestine diseases. However, regulation of transcription by FXR at genomic level is not known. This study analyzed genome-wide FXR binding in liver and intestine of mice treated with a synthetic FXR ligand (GW4064) by ChIP-seq. The results showed a large degree of tissue-specific FXR binding, with only 11% of total sites shared between liver and intestine. The sites were widely distributed between intergenic, upstream, intragenic, and downstream of genes, with novel sites identified within even known FXR target genes. Motif analysis revealed a half nuclear receptor binding site adjacent to the FXR response element, IR-1, indicating the involvement of other TFs for modulating FXR binding and/or function. Furthermore, pathway analysis indicated that FXR may be extensively involved in multiple cellular metabolic pathways. Conclusion: This study reports genome-wide FXR binding *in vivo* and the results clearly demonstrate tissue-specific FXR/gene interaction. In addition, FXR may be involved in regulating broader biological pathways in maintaining hepatic and intestinal homeostasis.

4.2: Introduction:

FXR, highly expressed in liver and intestine, is a ligand-activated TF belonging to the nuclear receptor superfamily. FXR was adopted when bile acids were identified as its endogenous ligands (Forman et al., 1995; Parks et al., 1999). FXR is essential in maintaining bile acid homeostasis and is important for energy balance through regulating lipid and glucose metabolism (Lambert et al., 2003; Ma et al., 2006; Sinal et al., 2000). FXR deficiency in mice has been implicated not only in hepatic and gastroenterological diseases, such as cholestasis, gallstones, NAFLD, liver and intestinal carcinogenesis, but also in systemic metabolic abnormalities such as atherosclerosis (Guo et al., 2006; Kong et al., 2009; Moschetta et al., 2004; Yang et al., 2007).

As a TF, FXR induces gene transcription by directly binding to an IR-1, as a heterodimer with the RXRα, in promoters of target genes including *Nr0b2*, *Abcb11*, *Ibabp*, *Osta* and *Ostb* (Laffitte et al., 2000). However, direct binding of FXR homodimer to promoter of *Apoa1*, encoding APOA-I, suppresses gene transcription (Claudel et al., 2002). FXR also suppresses gene transcription indirectly via induction of SHP (encoded by *Nr0b2*), an orphan nuclear receptor with only a ligand- but not a DNA-binding domain. SHP suppresses transcription through interactions with other TFs (Goodwin et al., 2000; Lu et al., 2000). FXR has emerged as a critical factor in mediating crosstalk between the liver and intestine to maintain bile acid, lipid and glucose homeostasis. Activation of FXR in the intestine induces Fgf 15/FGF 19 which travels to the liver to suppress the transcriptional activation of *Cyp7a1* that encodes a rate-limiting enzyme in bile acid synthesis (Inagaki et al., 2005; Kim et al., 2007; Song et al., 2009).

Despite the importance of FXR in regulating liver and intestine pathophysiology, a complete understanding of FXR-DNA interaction at the genomic level is not known. Several studies have identified transcriptional profiles in liver and intestine with FXR activation and/or deletion by microarray analysis (Downes et al., 2003; Inagaki et al., 2006; Xing et al., 2009). However, this approach cannot elucidate direct FXR binding. In addition, many *in vitro* FXR binding studies were performed in cell lines not of hepatic or intestinal origin. Instead, FXR was over-expressed in an artificial cellular environment and the promoters used to investigate FXR binding activity were outside of their natural chromatin context. Thus, many binding sites for a TF in living cells could be masked by the presence of a non-permissive chromatin environment, and therefore, the assays would reveal a false positive binding at these sites. New techniques have been developed to identify genomic binding sites of TFs. These techniques are ChIP followed by either the hybridization of the immunoprecipitated DNA pool to a tiling array (ChIP-chip) or by end-sequencing of millions of immunoprecipitated DNA fragments (ChIP-seq). ChIP-chip data tend to have low resolution and are often quite noisy (Johnson et al., 2008), and therefore, even with certain challenges, ChIP-seq is a better tool for analyzing genome-wide binding of TFs (Park, 2009). ChIP-seq has already been applied for quantitatively detecting nuclear receptor binding sites in a genome-wide manner (Nielsen et al., 2008).

In the current study, we have used ChIP-seq analysis to determine genome-wide FXR binding sites, in both the mouse liver and intestine. The results not only revealed novel binding sites for FXR, but also implicated new patterns of transcriptional regulation. This work highly suggests novel mechanism(s) by which FXR regulates

tissue-specific gene expression *in vivo* and reveals potential pathways to be regulated by FXR.

4.3: Results:

4.3.1: Determine the optimal time point of FXR/DNA binding *in vivo* following ligand treatment.

Due to the dynamic nature of TF-DNA binding, we first determined the optimal time point for FXR/DNA binding following treatment with GW4064 for 2, 4 or 8 hrs. In liver, FXR is known to bind to the promoter of the *Nr0b2* gene (Holt et al., 2003). Conversely, in intestine, FXR binds to FXRREs in the promoter regions of *Osta* and *Ostb* genes (Lee et al., 2006). Therefore, initial ChIP-qPCR assays were performed to amplify known FXRREs in *Nr0b2* (-320 to -220 bp upstream of TSS) for liver samples and *Osta* (-1245 to -1145 bp upstream of TSS) and *Ostb* (-220 to -150 bp upstream of TSS) for intestine samples. In both liver and intestine samples, binding of FXR to known FXRREs was strong and independent of GW4064 treatment when compared to untranscribed regions (Untr6), which serves as a negative control (**Figure 7a** and **7b**). This confirms the specificity of both the FXR binding and the FXR antibody. Based on these results, only samples from GW4064-treated mice were used for ChIP-seq analysis. Liver samples with 4 hr GW4064 treatment and intestine samples with 2 hr GW4064 treatment showed relatively stronger FXR binding, thereby were used for subsequent detection of FXR genome-wide binding sites by ChIP-seq.

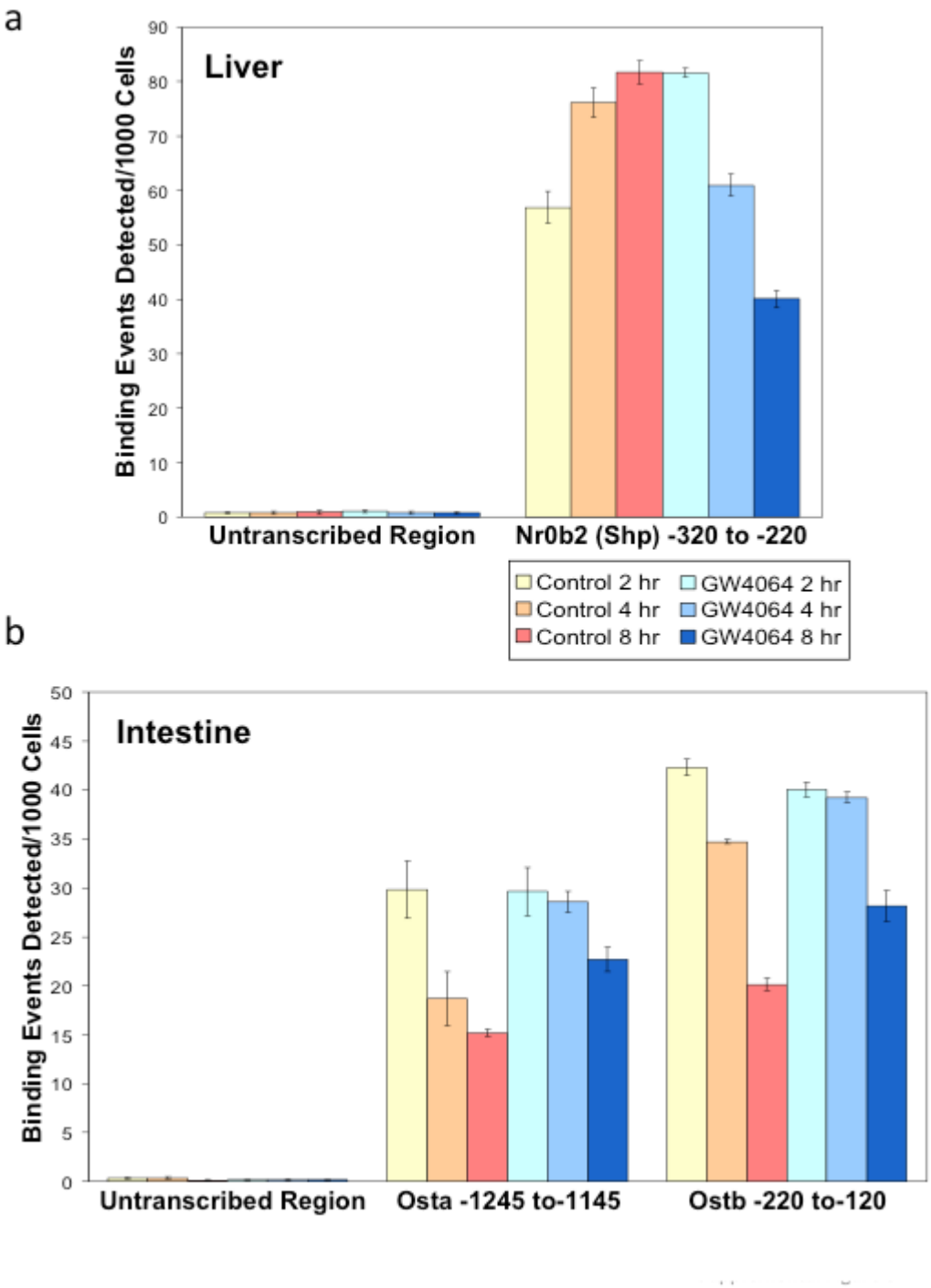
a
Liver
Binding Events Detected/1000 Cells
90
80
70
60
50
40
30
20
10
0
Untranscribed Region
Nr0b2 (Shp) -320 to -220
Control 2 hr
Control 4 hr
Control 8 hr
GW4064 2 hr
GW4064 4 hr
GW4064 8 hr
b
Intestine
Binding Events Detected/1000 Cells
50
45
40
35
30
25
20
15
10
5
0
Untranscribed Region
Osta -1245 to-1145
Ostb -220 to-120

Figure 7:

Figure 7: Initial validation of FXR binding to known FXRREs by ChIP-qPCR.

Predicted FXRREs are found at -320 to -220 bp upstream of *Nr0b2* TSS in the liver, -1245 to -1145 bp upstream of TSS of *Osta* and -220 to -150 bp upstream of *Ostb* in the intestine. Primers were designed to amplify these specific sites. The graphs are displayed as number of binding events detected per 1000 cells (y-axis) versus a specific amplified site (x-axis). a) Illustrates FXR binding at -320 to -220 bp upstream of *Nr0b2* in the liver of mice treated with vehicle or GW4064 for 2, 4, or 8 hrs. b) Illustrates FXR binding at -1245 to -1145 bp upstream of *Osta* and -220 to -150 bp upstream of *Ostb* in the intestine of mice treated with vehicle or GW4064 for 2, 4, or 8 hours. These ChIP-qPCR results show FXR binding at these sites is higher than for untranscribed regions of the genome (Untr 6). This indicates that the assay and the anti-FXR antibody used are specific to detect FXR bound regions of the genome. The greatest enrichment factor of GW4064 treated mice versus vehicle control is 1.59 (seen for *Osta* in the intestines for the 8 hr treatment group). These results indicate that FXR binding at these target sites is not sufficiently enhanced after treatment of animals with an FXR ligand. Therefore, only tissues from animals treated with GW4064 were used for ChIP-seq analysis.

Table 4: Previously reported FXR binding sites detected by ChIP-Seq.

Gene	Binding Site	Peak Value	Tissue
Nr0b2	-320 to -220 bp bp upstream of TSS	258; 124	liver; intestine
Abcb11	-240 to -140 bp upstream of TSS	308	liver
Fgf15	1880-1980 bp with in an intron	43; 273	liver; intestine
Osta	-1245 to -1145 bp upstream of TSS	502	intestine
Ostb	-220 to -150 bp upstream of TSS	271; 572	liver; intestine
Fabp6	site 1: -220 to -120; site 2: -2990 to -2890; and site 3: -7600 to -7500 bp upstream	540, 638, 93	intestine

4.3.2: Validation of FXR binding sites discovered by ChIP-seq.

To validate the ChIP-seq results, novel FXR binding sites were randomly chosen for confirmation by ChIP-qPCR assay. In the ChIP-seq data set, the intensity of FXR binding to DNA was reported as peak value. Many known FXRREs previously identified were detected with relatively high peak values (**Table 4**). Based on peak values reported in ChIP-seq data, the sites listed in Table 4, together with other randomly selected novel sites, were categorized as having high (>300), medium (100-300), or low (<100) peak values and were tested by ChIP-qPCR, with gene names listed in **Tables 5** and **6**. These sites were tested in liver only (20 sites), intestine only (5 sites), or in both tissues (27 sites). The results showed 100% of the sites with high, 84.4% with medium

Table 5: List of genes validated after the ChIP-seq assay in the liver.

Peak Value	Genes Validated	Genes not validated
> 300 peak value	Hmga1, Fcna (1), Fcna (2), Slc10a1 (2), Slc9a8, Pdgfrb, Pddc1, Sumo3, Tcfap2e, Cks2, Mirn126 (2), Nr0b2 (1), Nr0b2 (2), Ostb	
100-300 peak value	Mir499, Rarres2, Stat2, Caprin1, Cdk5rap2, Actg1, Vkorc1, Mirn126 (1), Cnnm3 (1), Nat2, IL17b, Pcx (1), Ldha (2), Bccip	Rorc, Smurf1, Aldh5a1, Rdh7, Apoe (1)
<100 peak value	Cebpb, Gtf2a2, Ell2, Fga-(1), Igfals, Tlr3, Trmp7, Gpbp1 (1)	Cdh 15, Slc10a1 (1), Fga (2), Ldha (1), Cnnm3 (2)

Note: (1) and (2) indicate two sites identified in one gene.

Table 6: List of genes validated after the ChIP-seq assay in the intestine.

Peak Value	Genes Validated	Genes not validated
> 300 peak value	Slc25a3, Slc22a21, Slc9a8, Pddc1, Cks2, Tlr3, Pcx (1), Ldha (2), Mirn126 (2), Fgf15, Nr0b2 (2), Osta, Ostb	
100-300 peak value	Plat, Nr0b2 (1), Actg1, Cdk5rap2, Sumo3, Caprin1, Mirn126 (2), Cnnm3 (1), Nrld2, Vkorc1, IL17b, Apoe (1), Ldha (1)	
<100 peak value	Pdgfrb, Tcfap2e, Nat2, Trpm7, Bccip	Mycbp

Note: (1) and (2) indicate two sites identified in one gene.

Table 7: Validation of ChIP-seq: percentage of ChIP-seq binding sites confirmed by ChIP-qPCR.

	Peak Value	%Confirmation	
27 genes	>300	100	(27/27)
32 genes	100-300	84.4	(27/32)
20 genes	<100	70	(14/20)

and 70% with low peak values were confirmed (**Table 7**). All together, 86% (68/79) of the sites were verified by ChIP-qPCR as true FXR binding sites. Based on these results, we concluded the ChIP-seq results are relatively accurate. Therefore, more detailed analysis of the ChIP-seq results was performed.

4.3.3: Analysis of genome-wide FXR binding sites in liver and intestine.

There were a total of 7794 FXR binding sites in liver and 5321 in intestine. However, 6345 sites were found specifically in liver, 3872 specifically in intestine, and 1449, representing 11% of total sites, were bound by FXR in both tissues (**Figure 8a**). The FXR binding sites were widely distributed throughout the mouse genome. Forty-one % of total FXR binding sites in liver, and 39% in intestine, were located more than 10 kb upstream of a RefSeq gene (intergenic regions). Conversely, 59% of the total sites in liver and 61% in intestine were found directly associated with a gene, indicating that FXR binding in both liver and intestine is more highly concentrated within coding regions of the mouse genome. Of these binding sites: 1% in liver and intestine overlap with 5'-untranslated regions (5'UTRs); 4% in liver and 3% in intestine were located in exon regions; 29% in liver and 31% in intestine were located in intron regions; less than

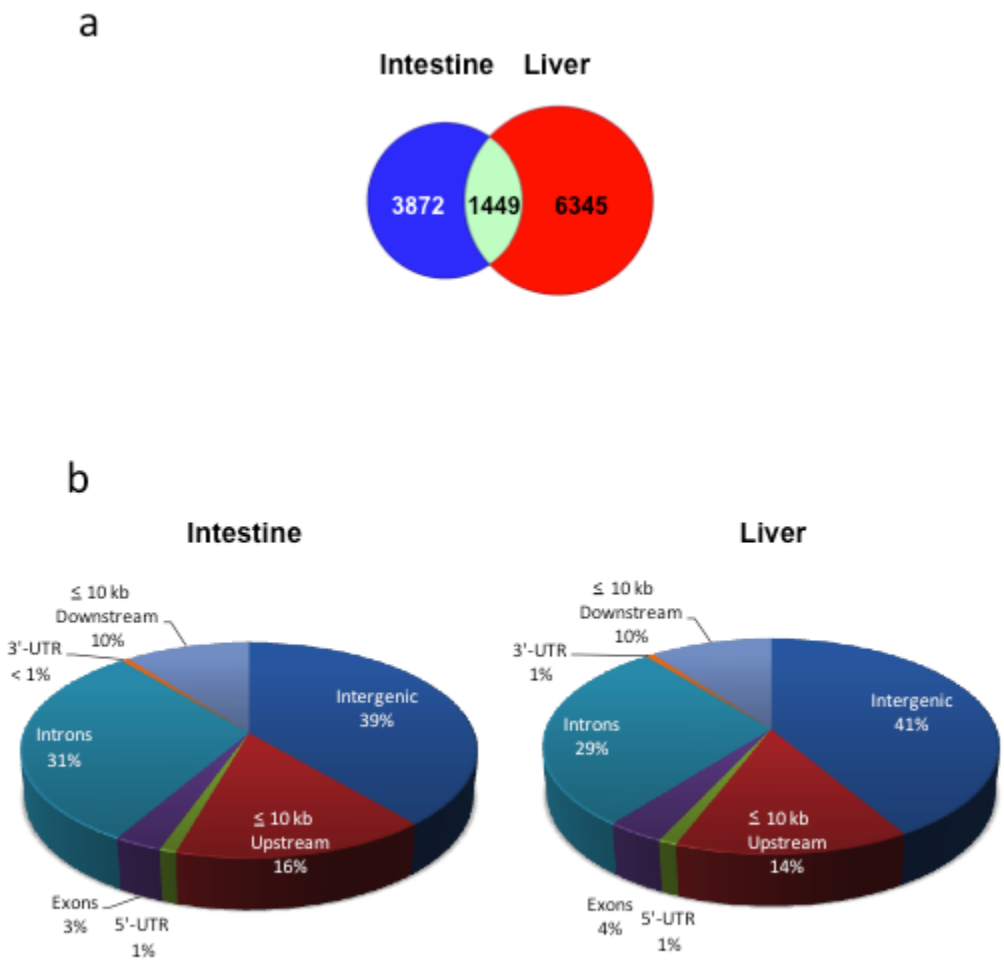
a
Intestine
Liver
3872
1449
6345
b
Intestine
≤ 10 kb Downstream 10%
3'-UTR < 1%
Intergenic 39%
Introns 31%
≤ 10 kb Upstream 16%
Exons 3%
5'-UTR 1%
Liver
≤ 10 kb Downstream 10%
3'-UTR 1%
Intergenic 41%
Introns 29%
≤ 10 kb Upstream 14%
Exons 4%
5'-UTR 1%

Figure 8:

Figure 8: Total FXR binding sites in liver and intestine. a) A venn diagram of total FXR binding in liver (red), intestine (blue), or both (green). b) Percentage of FXR binding sites in liver and intestine that were distributed to >10 kb from genes (intergenic), < 10 kb upstream of genes, introns, exons, 5'UTRs, 3'UTRs, and < 10 kb downstream of a genes.

1% in liver and intestine overlap with 3'UTRs; and 10% in liver and intestine were located within10 kb downstream of genes (**Figure 8b**).

Many genes were bound by FXR at multiple regions, including upstream, in-gene, and downstream of genes, including known FXR target genes previously identified to have only one FXRRE. For example, multiple FXR binding sites were identified for *Nr0b2* and *Ostb* (**Figure 9a and 9b**). Therefore, the total number of genes to which FXR bound was less than the total number of binding sites. There were 4248 genes in liver and 3406 genes in intestine to which FXR was shown to bind at a single or multiple regions. Among them, 1713 genes were shared by both liver and intestine.

The average peak value of FXR binding sites was higher for sites within 10 kb upstream of TSSs, and the frequency of binding (number of binding events) was higher for binding sites located near the TSS of target genes in both liver and intestine (**Figure 10a**). The average peak value decreased with distance from proximal promoter and frequency of binding decreased with the distance from the TSSs. In addition, there was a relatively high percentage of intron binding of FXR (**Figure 8b**). Therefore, the genome-wide intron binding of FXR was further evaluated for cumulative binding events of FXR within intron regions in the liver and intestines (**Figure 10b**). Most of the intron binding of FXR was in close proximity to TSSs of target genes, with 1107 peaks from both tissues falling within the first intron of a gene [640 peaks in the liver (red) and 467 peaks in the intestine (blue)].

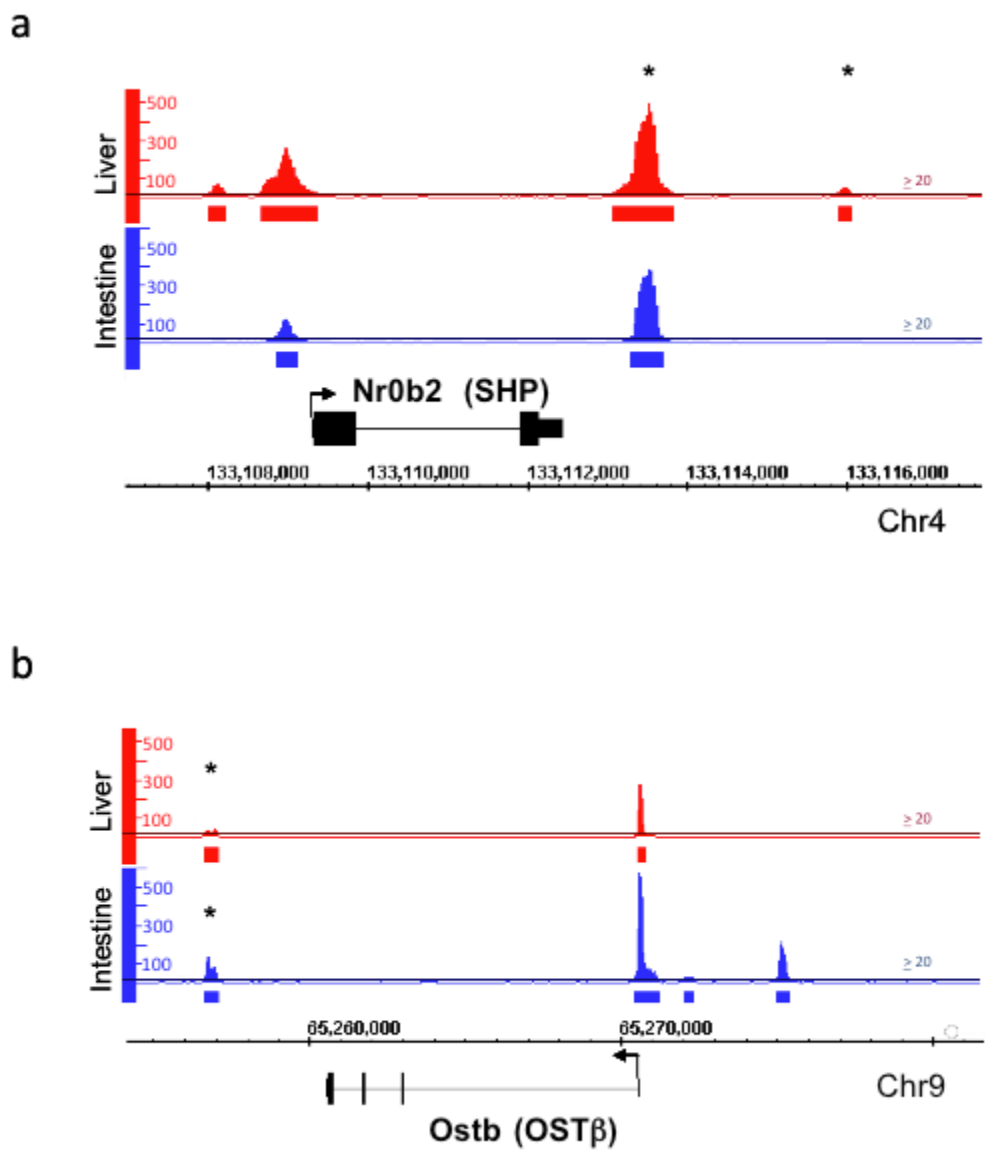
a
Liver
Intestine
500
300
100
≥ 20
*
*
Nr0b2 (SHP)
133,108,000
133,110,000
133,112,000
133,114,000
133,116,000
Chr4
b
Liver
Intestine
500
300
100
≥ 20
*
*
65,260,000
65,270,000
Chr9
Ostb (OSTβ)

Figure 9:

Figure 9: Histogram of novel FXR binding sites within known FXR target genes, *Nr0b2* and *Ostb*. The y-axis displays the peak value and the x-axis shows the chromosomal location of the gene. The top panel (displayed in red) represents FXR binding in liver, and the bottom panel (displayed in blue) represents FXR binding in intestine. Genes that are displayed above the chromosome scale are oriented in the sense directions (right arrow), and genes that are displayed below the chromosome scale are oriented in the anti-sense direction (left arrow). The threshold is set at peak value > 20. a) The *Nr0b2* gene is located on chromosome 4 (Chr4): 133109305-133112451 and is oriented in the sense direction in this figure. FXR binds at 2 locations around the *Nr0b2* gene in intestine, promoter region (peak value = 124) and 3' end of the gene (peak value = 381). FXR binds at 4 locations of the *Nr0b2* gene in liver, 2 in the promoter and 2 at the 3' end (peak values are 69, 258, 498, and 49, respectively). b) The *Ostb* gene is located at Chr9: 65260560-65270580 and is oriented in the anti-sense direction. In intestine, FXR binds at 4 locations near the *Ostb* gene, with 3 sites within the promoter (peak value = 572, 27, and 210) and 1 located at the 3' end (peak value= 132). In liver, FXR binds once within the promoter (peak value = 271) and once at the 3' end (peak value = 44). Novel FXR sites found at the 3' end of both *Nr0b2* and *Ostb* gene have not been reported and are indicated by '*.'

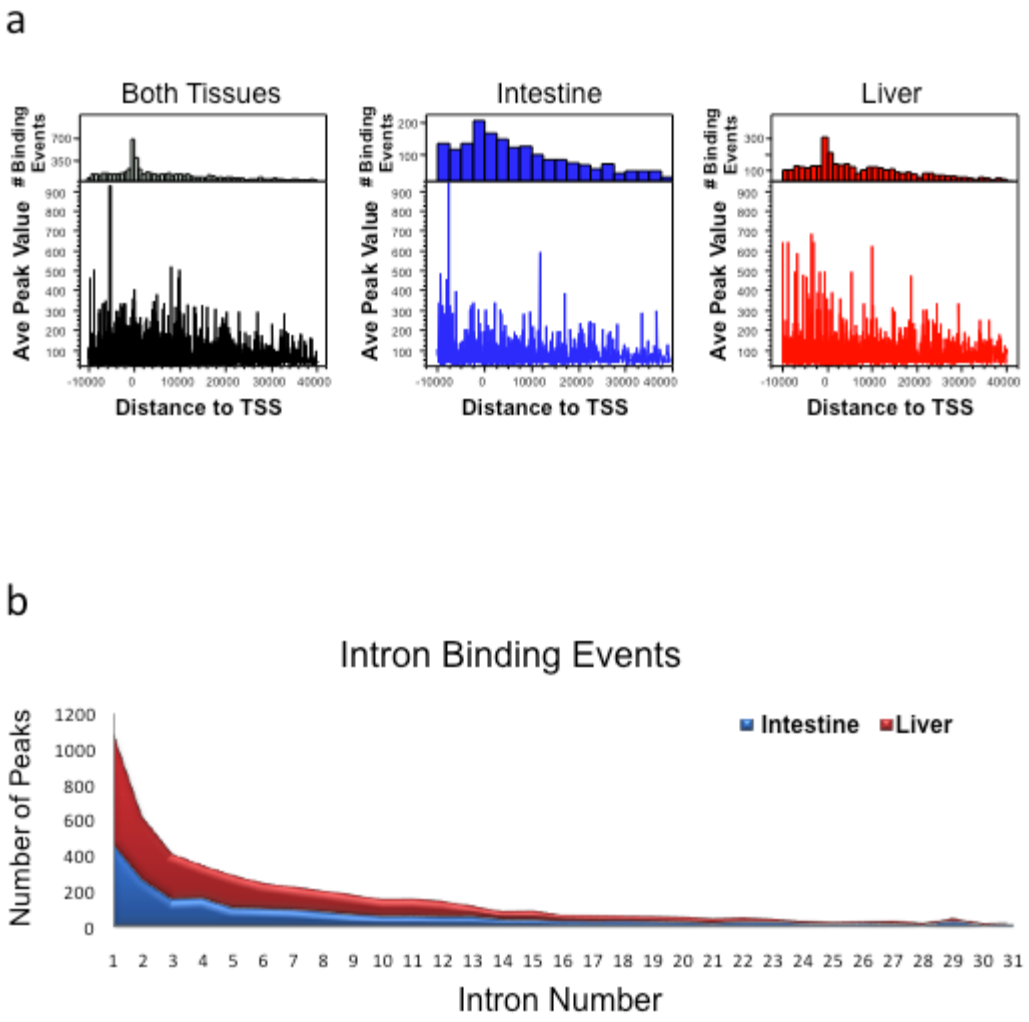
a
Both Tissues
Intestine
Liver
Binding Events
Ave Peak Value
Distance to TSS
b
Intron Binding Events
Intestine
Liver
Number of Peaks
Intron Number

Figure 10:

Figure 10: Distribution of total FXR binding sites relative to TSSs and intron binding of FXR. a) The top panel is the frequency distribution of FXR binding, showing the number of binding events (y-axis) at each distance from TSSs (x-axis). The bottom panel displays average peak value of binding sites at each distance from TSSs. The y-axis displays the average peak value and the x-axis shows the distance of binding site from the TSS. The highest number of FXR binding events was greatest at the TSSs of genes, and the average peak value of FXR binding sites was greatest within 10 kb upstream of TSSs of genes. b) The cumulative binding events of FXR distributed only to introns of genes in the liver (red) and intestine (blue). The graph displays the total number of FXR binding peaks (y-axis) in the liver and intestine located within intron 1-31 of genes (x-axis). There were a higher number of peaks within the first intron of genes with 1107 total peaks within first intron for both liver and intestine.

4.3.4: Novel FXR binding sites revealed by ChIP-Seq.

FXR was found to bind to many novel sites within the mouse genome. For example, FXR bound to several sites within the first intron of the *Nr1i2* gene, which encodes PXR (highest peak value: 654 in liver and intestine, indicated by '*'; **Figure 11a**). A novel FXR binding site was also discovered at a 3' regulatory region of the *Nr0b2* gene (peak value: 498 in liver and 381 in intestine, indicated by '*'; **Figure 9a**). Binding of FXR to this site in the liver has been confirmed by regular ChIP-qPCR (**Figure 5b**).

FXR has been shown to up-regulate both OSTα and β (encoded by *Osta* and *Ostb* respectively) in liver and intestine (Boyer et al., 2006; Lee et al., 2006). This has been previously recorded as being a result of direct FXR binding to regulatory regions of *Osta* and *Ostb* genes in both liver and intestine (Landrier et al., 2006). The current study confirmed the binding of FXR in intestine to upstream regions of *Osta* and *Ostb* (**Figure 11b**: *Osta* and **Figure 9b**: *Ostb*). In addition, in liver FXR bound to upstream regions of *Ostb* (**Figure 9b**). However, these results clearly showed that FXR did not bind to regulatory regions of *Osta* in liver (**Figure 11b**). Furthermore, binding of FXR to 3' end of *Ostb* and to the proximal promoter and intron region of *Osta* in the intestine has not been previously reported (indicated by '*'). The present results also showed FXR bound directly to two upstream regulatory regions of *Slc10a1*, which encodes Ntcp, in liver with relatively high intensity (peak value: 50 at -150 to -50 bp and 300 at -8100 to -8000 bp), which are uncharacterized FXR binding sites (indicated by '*,' **Figure 11c**). Ntcp is a bile acid uptake transporter essential for hepatic uptake of conjugated bile acids. ChIP-seq results on FXR binding to *Osta*, *Ostb*, and *Slc10a1* were confirmed by ChIP-qPCR

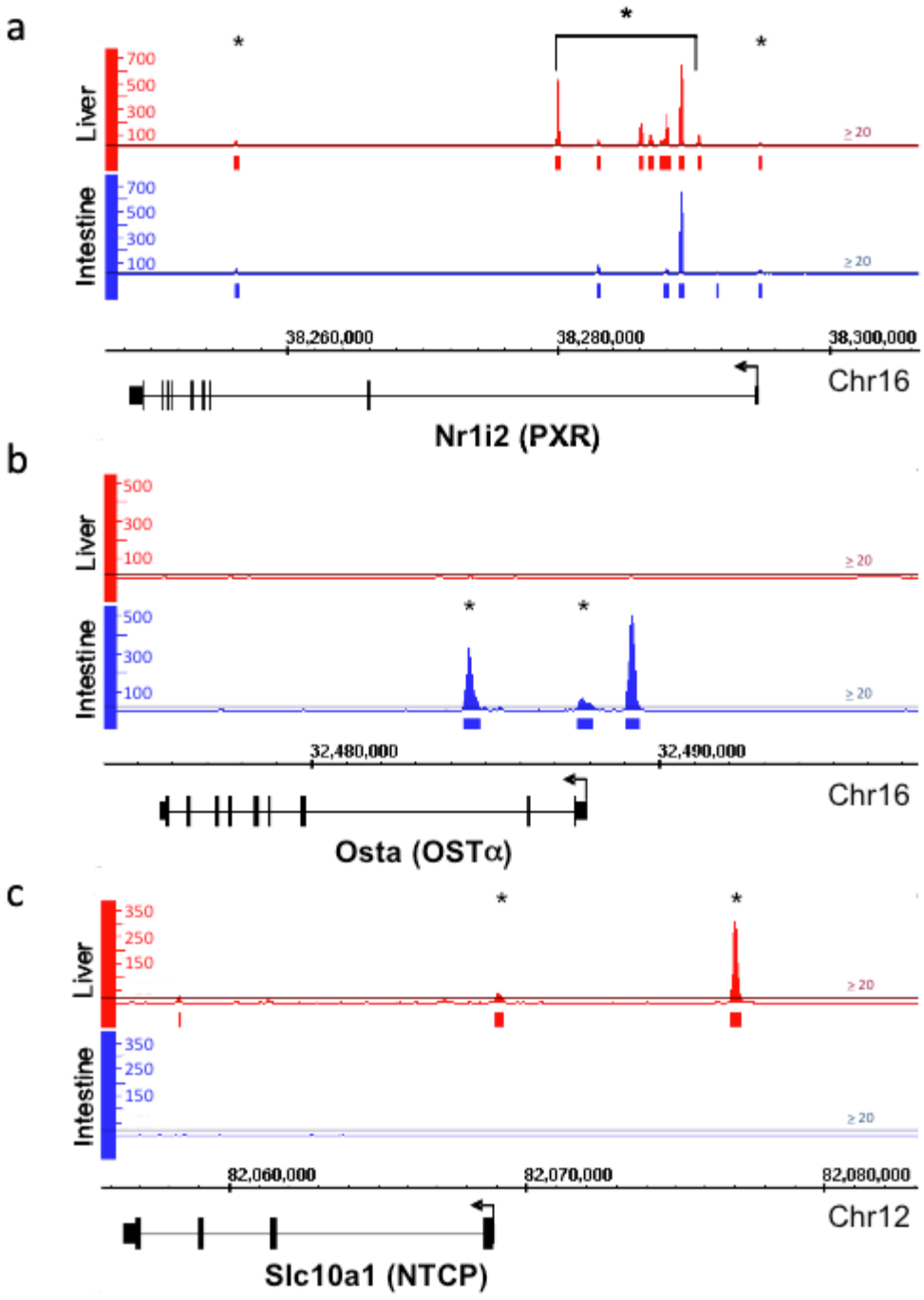
a
Liver
Intestine
700
500
300
100
≥ 20
38,260,000
38,280,000
38,300,000
Chr16
Nr1i2 (PXR)
b
Liver
Intestine
500
300
100
≥ 20
32,480,000
32,490,000
Chr16
Osta (OSTα)
c
Liver
Intestine
350
250
150
≥ 20
82,060,000
82,070,000
82,080,000
Chr12
Slc10a1 (NTCP)

Figure 11:

Figure 11: Histogram of novel FXR binding sites within *Nr1i2*, *Osta*, and *Slc10a1*.

a) The *Nr1i2* gene is located on Chr16: 38248437-38294909 and is oriented in the anti-sense direction. Previously uncharacterized FXR binding sites were found at multiple regions within the *Nrli2* gene indicated by '*' (the brackets include all FXR binding sites in the 1st intron of the gene). These binding sites were located within the promoter region and introns 1 and 2 of the gene (highest peak value was 646 in liver and 654 in intestine). b) The *Osta* gene is located on Chr16: 32475664-32487965 and is oriented in anti-sense direction. FXR binds to *Osta* in the intestine but not in the liver, displaying tissue-specific binding. Binding of FXR to promoter region (-1245 to -1145 upstream of TSS) of *Osta* in the intestine has been previously characterized, and serves as a positive control for our analysis (peak value = 502). However, binding of FXR within -100 bp upstream of TSS (peak value = 70) and within intron 2 of *Osta* in the intestine are novel findings (peak value = 333; indicated by '*'). c) The *Slc10a1* gene is located on Chr12: 82056479-82068971 and is oriented in the anti-sense direction. FXR binds at 2 sites in the *Slc10a1* promoter in liver (around -100 bp and -8000 bp upstream of TSS, peak value = 38 and 304, respectively; indicated by '*'). Binding of FXR to these sites were confirmed by ChIP-qPCR (data not shown).

(data not shown). The histograms of *Osta* and *Slc10a1* clearly show tissue-specific DNA binding of FXR, with *Osta* only being bound in intestine (**Figure 11b**) and *Slc10a1* only being bound in liver (**Figure 11c**).

4.3.5: Motif analysis of FXR binding sites.

As mentioned earlier, the most characterized FXRRE is an IR-1. This motif has been found within many known FXR target genes, including *Nr0b2, Fabp6* (encodes Ibabp) and *Fgf15/FGF19*. In the current study, the most common motif identified was an IR-1 (**Figure 12**). In this analysis, the different sized nucleotides represent the probability of the represented nucleotide being located at a specific location. The larger nucleotides represent a higher probability of the motif containing that nucleotide sequence and therefore are considered more significant. Interestingly, a half nuclear receptor binding site, AGGTCA, was found adjacent to the IR-1 at the 3' end in the liver. Conversely, the most commonly occurring sequences for intestine FXR binding sites were an IR-1 and an everted repeat separated by 2 nucleotides (ER-2). As with the liver samples, there was a half site adjacent to the FXR binding sites in intestine. However, the half site could be at the 3' end (TGACCT) if FXR binding site was an ER-2 or at the 5' end (TGACCT) if FXR binding site was an IR-1. Nevertheless, the motif analysis suggests tissue-specific sequence motifs to which FXR recognizes and binds.

4.3.6: Biological pathway analysis.

FXR is essential in regulating pathways important for bile acid, lipid, and glucose homeostasis. However, it is highly likely that this nuclear receptor may be involved in

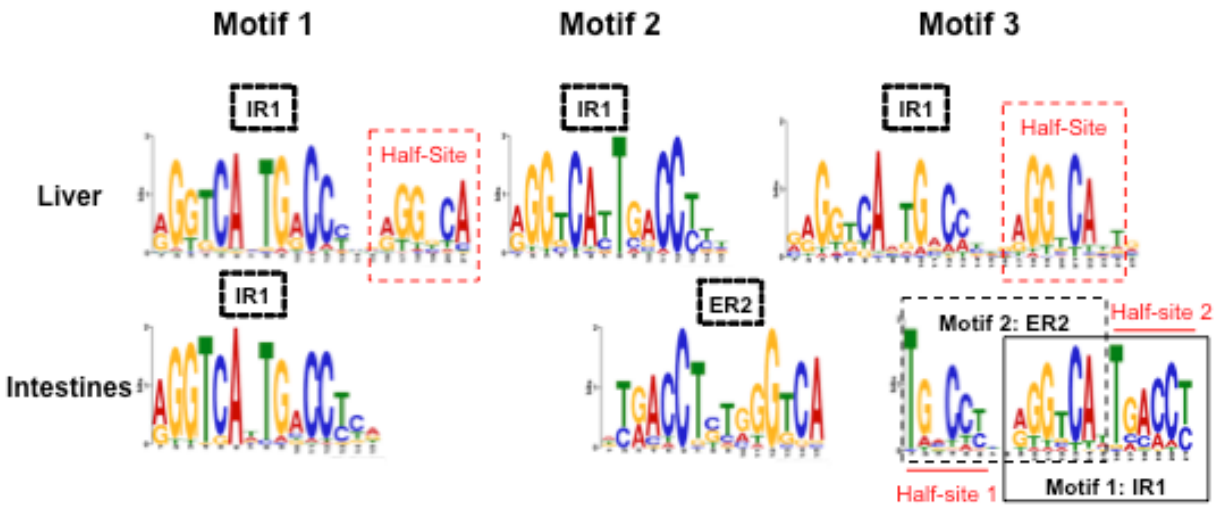
Motif 1
Motif 2
Motif 3
Liver
Intestines
IR1
Half-Site
ER2
Motif 2: ER2
Half-site 2
Half-site 1
Motif 1: IR1

Figure 12:

Figure 12: Motif analysis. Three most commonly identified sequence motifs of the top 500 FXR binding sites in the liver (top panel) and intestine (bottom panel) are shown according to MEME analysis. In the liver, an IR-1 was the most common sequence motif found. Two of the three motifs identified (left and right motif) in the liver showed the presence of a half nuclear receptor binding site at the 3' end of the IR-1. In the intestine, an IR-1 (left motif) or an ER-2 (middle motif) were motifs commonly found within the top 500 FXR binding sites. The motifs found in the intestine are arbitrarily aligned to match up with the sequence motifs in the liver to show sequence similarity. In the intestine, a half nuclear receptor binding site was also associated with the identified FXRRE either at the 5' end (half site 1) or at the 3' end (half site 2), depending on which motif is recognized as a FXRRE. E-values, a measure of significance, were between 3.8e-037 to 9.3e-085.

Table 8: Pathways enriched with FXR binding 2 kb up-stream of genes in liver.

Tissue	Biological Process	Genes*	% bound by FXR	P-Value	Bonferroni
liver	primary metabolic process	92	93.9	1.70E-25	8.80E-22
	cellular metabolic process	88	89.8	1.90E-20	9.60E-17
	biosynthetic process	38	38.8	7.60E-16	4.00E-12
	cellular biosynthetic process	25	25.5	5.60E-09	2.90E-05
	nitrogen compound metabolic process	25	25.5	4.90E-17	2.60E-13
	amine metabolic process	24	24.5	1.40E-16	5.80E-13
	amino acid and derivative metabolic process	24	24.5	3.80E-18	2.00E-14
	amino acid metabolic process	23	23.5	3.20E-19	1.70E-15
	lipid biosynthetic process	21	21.4	8.90E-17	5.80E-13
	steroid metabolic process	18	18.4	7.50E-17	5.80E-13
	monosaccharide metabolic process	11	11.2	3.80E-08	1.90E-04
	hexose metabolic process	11	11.2	3.10E-08	1.60E-04
	sterol metabolic process	11	11.2	4.50E-11	2.30E-07
	cholesterol metabolic process	11	11.2	1.70E-11	8.80E-08
	glucose metabolic process	10	10.2	2.80E-08	1.50E-04
	lipid catabolic process	10	10.2	2.40E-08	1.20E-04
	fatty acid oxidation	8	8.2	1.30E-10	7.00E-07
	steroid biosynthetic process	7	7.1	6.30E-06	3.20E-02
	glycine, serine and threonine metabolism	7	7.1	1.20E-04	2.30E-02
	isoprenoid metabolic process	7	7.1	8.70E-08	4.50E-04
	pyruvate metabolic process	7	7.1	8.40E-09	4.30E-05
	pyruvate metabolism	6	6.1	5.60E-04	1.00E-01
	amino acid biosynthetic process	6	6.1	7.80E-06	4.00E-02
	glycerol ether metabolic process	6	6.1	1.30E-06	6.80E-03
	glycerolipid metabolic process	6	6.1	1.10E-06	5.70E-03
	neutral lipid metabolic process	6	6.1	1.10E-06	5.70E-03
	acylglycerol metabolic process	6	6.1	9.20E-07	4.80E-03
	sterol transport	6	6.1	9.50E-08	4.90E-04
	cholesterol transport	6	6.1	7.00E-08	3.60E-04
	triacylglycerol metabolic process	5	5.1	9.80E-06	4.90E-02
	gluconeogenesis	5	5.1	6.50E-06	3.30E-02
	cholesterol homeostasis	5	5.1	5.20E-06	2.70E-02
	lipid homeostasis	5	5.1	5.20E-06	2.70E-02
	sterol homeostasis	5	5.1	5.20E-06	2.70E-02

Note: * indicates the number of genes bound by FXR in this pathway

other biological pathways not been previously recognized. To further determine biological pathways that may be regulated by FXR, genes bound by FXR less than 2 kb upstream of their TSSs were selected for biological pathway analysis. Functional Annotation analysis revealed FXR may be involved in several metabolic pathways in liver (**Table 8**). In fact, 90% of genes categorized into the cellular metabolism process were bound by FXR in the liver. Furthermore, FXR may also be significantly involved in regulating other pathways that are not known to be regulated by FXR, such as amino acid and nitrogen compound metabolism.

In intestine, however, FXR seems to regulate different pathways than in liver. For example, FXR binds to 40% of the genes categorized in the catalytic pathway processes, suggesting FXR may be highly involved in regulating catalytic pathways in the intestine. In addition, pathways involved in oxidoreductase activity, monooxygenase activity, and cofactor binding seemed to be highly enriched with FXR binding in intestine (**Table 9**). Interestingly, pathways involved in intestinal inflammation, such as complement and coagulation cascades, are also enriched with FXR binding.

4.4: Concluding Remarks:

In the current study, genome-wide FXR binding sites were analyzed by ChIP-seq analysis in both liver and intestine following treatment of mice with a potent synthetic FXR ligand. The current study not only summarizes binding sites for FXR within the mouse genome in liver and intestine, but also suggests potential tissue-specific patterns of transcriptional regulation mediated by FXR.

Table 9: Pathways enriched with FXR binding 2 kb up-stream of genes in intestine.

Tissue	Biological Process	Genes*	% bound by FXR	P-Value	Bonferroni
intestine	catalytic activity	283	40.8	2.20E-16	6.00E-13
	oxidoreductase activity	64	9.2	2.30E-07	6.20E-04
	generation of precursor metabolites and energy	46	6.6	2.40E-07	1.20E-03
	electron transport	36	5.2	9.90E-06	5.00E-02
	iron ion binding	30	4.3	3.10E-06	8.40E-03
	cofactor binding	24	3.5	1.30E-07	3.40E-04
	vitamin binding	19	2.7	1.70E-08	4.60E-05
	monooxygenase activity	17	2.4	1.00E-05	2.80E-02
	complement and coagulation cascades	14	2	9.70E-05	1.90E-02
	unspecific monooxygenase activity	9	1.3	2.10E-05	5.50E-02

Note: * indicates the number of genes bound by FXR in this pathway

The initial validation study by regular ChIP-qPCR assay showed that *in vivo* FXR appears to bind to its response element in the regulatory region of FXR target genes, presumably due to activation by endogenous ligands (bile acids). The binding intensity has been slightly enhanced upon treatment with a potent synthetic ligand, GW4064. Thereby treatment with GW4064, a ligand with higher affinity for FXR than bile acids (Maloney et al., 2000), may result in an endogenous-to-synthetic ligand switch. In agreement with this notion, previous *in vitro* studies using HepG2 cells, which are known to synthesize bile acids, have shown that FXR binds to its response element weakly in the presence of control vehicle, and treatment with various synthetic ligands enhanced the binding (Fang et al., 2008). Furthermore, we also observed a weak FXR interaction with its response element in the mouse hepatoma cell line, Hepa1c1c cells,

and this interaction was enhanced upon treatment with GW4064 (data not shown). Collectively these results may indicate a ligand switch for FXR.

To further validate the ChIP-seq data, we have also applied a different peak finding algorithm to our data set. We have been using MACS (Zhang et al., 2008). It looks for local enrichments of alignments that show the correct orientation that would be expected for a ChIP peak (peak modeling). It thus eliminates at least some false positives. The analysis from the MACS showed that of the 7796 liver peaks (intervals) in the original analysis, 6942 (89.05%) were also identified by MACS, and of the 5324 original Ileum peaks, 5023 (94.35%) were also identified by MACS.

Using the ChIP-seq discovery approach, DNA regions bound by FXR have been identified throughout the entire mouse genome. This study shows a novel FXR binding pattern with novel FXR binding sites located large distances upstream (intergenic), downstream, or within genes (intragenic). In fact, this study shows a large portion of FXR binding sites are located more than 10 kb away from a RefSeq gene. This indicates long-distance chromatin interactions as a possible mechanism for FXR-mediated regulation of gene transcription (Li et al., 2004). However, the majority of FXR binding sites are concentrated within coding regions of the mouse genome. Of these sites, FXR is highly associated with the proximal promoter and first intron. Recent ChIP-tiling array and ChIP-seq reports on other nuclear receptors and TFs have also revealed a high percentage of intergenic binding (Gao et al., 2008; Lupien et al., 2008; Nielsen et al., 2008). In addition, a previous report of genome-wide ERα binding has also shown a higher intensity of binding near the TSSs of genes, which is consistent with this study (Gao et al., 2008).

This study exhibits a high degree of tissue-specific binding sites for FXR as well as a tissue-specific binding pattern, with a relatively low portion of total sites shared between liver and intestine. For example, *Nr0b2*, a classical FXR target gene encoding SHP, is expressed at a much higher level in liver than in intestine (Kamisako et al., 2007). In both tissues, SHP expression levels can be strongly induced through activation of FXR. The current study reveals that FXR binds with high intensity to a downstream IR-1 of the *Nr0b2* gene in both liver and intestine. Furthermore, FXR also binds to other known target genes, including *Osta*, *Ibabp*, and *Slc10a1* in a tissue-specific manner. *Ibabp* and *Osta* are bound by FXR in intestine but not in liver, which, for *Osta*, is in contrast with a previous report that FXR also binds to the promoter region of *Osta* in liver (Boyer et al., 2006). Likewise, the *Slc10a1* gene, encoding Ntcp, has two FXR binding sites within promoter regions in liver. Ntcp has been shown to be suppressed by high concentrations of bile acids under cholestatic conditions through an FXR-SHP mediated mechanism (Zollner et al., 2005). However, the finding of FXR binding sites in the *Slc10a1* promoter region suggests that FXR may directly regulate the transcription of the *Slc10a1* gene. Furthermore, tissue-specific FXR regulation of these genes may be mediated by tissue-specific cofactors or chromatin modification.

The motif analysis indicates the most represented FXR binding motif in mouse liver is an IR-1, which has been indentified in many FXR target genes. In mouse intestine, an IR-1 was also the most commonly represented motifs; however, an ER-2 motif was also represented as a possible FXR binding sequence. In addition, there was a half nuclear receptor binding site adjacent to the FXR binding site in both liver and intestine. The motif analysis suggests there are tissue-specific sequence motifs to which

FXR recognizes and binds. In addition, this new finding indicates a potential interaction between FXR and other orphan nuclear receptors/TFs known to bind to this half site (Ito et al., 2000). These cofactors may be important for modulating transcriptional activation of FXR target genes. The interaction between hormone nuclear receptors and other TFs have been previously reported. For example, ERα has been shown to interact with FOXA1 for optimal DNA binding and transcriptional activation (Lupien et al., 2008).

The current study also suggests that FXR may directly regulate more diverse biological pathways. For example, FXR binds to *Rara* and *Rorc* and may regulate transcriptional activation of these genes as well as retinol and hormone metabolism (data not shown). This means that FXR not only regulates bile acid and lipid homeostasis, but may also be directly involved in other cellular functions. In addition, FXR may regulate bile acid and lipid homeostasis to a greater extent than previously determined. Further studies are needed to characterize the significance of FXR's involvement in these pathways to clarify networks controlled and shared by FXR to maintain cellular and organ homeostasis.

In summary, this study analyzed genome-wide FXR-DNA binding in both the mouse liver and intestine following ligand treatment. The identification of novel FXR binding sites, as well as unique binding patterns, enable a more profound understanding of FXR-regulated biological and disease pathways in a tissue-specific manner.

Chapter 5: FXR and HNF4α Interact to Cooperatively Regulate Gene Transcription in the Liver

5.1: Abstract:

HNF4α is a nuclear receptor critical for regulating liver development, differentiation and function. FXR is also a ligand-activated nuclear receptor critical for liver function. Studies show mice deficient in FXR develop cholestasis, hyperlipidemia and liver tumors. The old paradigm suggests linear activation of target gene transcription following direct binding of FXR to gene regulatory regions. However, we showed that FXR activated gene transcription by cooperating with HNF4α to regulate gene transcription in the liver. Data obtained from ChIP-seq analysis of mouse livers showed nearly 50% of FXR binding sites in liver overlapped with HNF4α binding sites. In addition, the majority of FXR and HNF4α binding sites were close in proximity. Co-IP assays imply that FXR may directly interact with HNF4α. Furthermore, luciferase assays suggest an interaction of HNF4α and FXR leads to a modest additive effect on FXR-mediated transcriptional activation. In conclusion, this study shows initial evidence of a cooperative interaction between FXR and HNF4α in regulating liver gene transcription.

5.2: Introduction:

FXR is a ligand-activated TF belonging to the nuclear receptor superfamily. FXR is classified as a group II nuclear receptor and bile acids are its endogenous ligands (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). FXR is highly expressed in the liver and intestine and is a master regulator of the enterohepatic circulation of bile acids at the transcriptional level (Kok et al., 2003; Sinal et al., 2000; Zhang et al., 2003). FXR has also been shown to regulate other metabolic processes such as lipid homeostasis, glucose metabolism, insulin sensitivity, and liver/colon cancer development, and therefore is a potential therapeutic target for the treatment or prevention of cholestasis, hyperlipidemia, fatty liver, type II diabetes, and liver and colon cancer (Cariou et al., 2006; Claudel et al., 2003; Kim et al., 2007; Ma et al., 2006; Modica et al., 2008; Staels and Kuipers, 2007; Watanabe et al., 2004; Yang et al., 2007; Zhang et al., 2006). Recent genome-wide binding studies have shown that FXR displays a high degree of tissue-specific binding (Thomas et al., 2010). In addition, motif analysis of genome-wide FXR binding in liver revealed a half nuclear receptor binding site associated with the FXRRE, which is an IR-1 (Chong et al., 2010; Thomas et al., 2010). These studies suggest the involvement of other orphan nuclear receptors in regulating FXR function.

HNF4α is a highly conserved orphan nuclear receptor and linoleic acid has been shown to occupy the LBD of HNF4α (Sladek et al., 1990; Yuan et al., 2009) but does not affect transcriptional activity of HNF4α (Yuan et al., 2009). HNF4α is critical for liver development, differentiation, and organism survival (Chen et al., 1994). In hepatocytes, HN4α localizes mainly to the nucleus, binds DNA exclusively as a homodimer, and

recognizes response elements consisting namely of DR-1 (Gonzalez, 2008). HNF4α regulates the expression of a myriad of liver-specific genes including production of clotting factors, apolipoprotein synthesis, and drug metabolism (Gonzalez, 2008). In addition, HNF4α has been shown to directly regulate the transcription of Cyp7a1 and Cyp8b1, enzymes responsible for bile acid synthesis, as well as the expression of other genes critical for bile acid metabolism suggesting HNF4α is also important in regulating bile acid homeostasis (Crestani et al., 1998; Inoue et al., 2006; Stroup and Chiang, 2000). In fact, hepatocyte-specific deletion of HNF4α in mice leads elevated serum bile acid levels (Hayhurst et al., 2001).

FXR and HNF4α have already been shown to regulate the expression of the same genes such as apolipoprotein C-III (APOC-III), CYP7A1, and bile acid-CoA: amino acid N-acyltransferase (BAAT; Claudel et al., 2003; Inoue et al., 2004; Inoue et al., 2006; Shih et al., 2000), thereby, suggesting an overlap of FXR and HNF4α function in the liver. However, a study investigating how FXR and HNF4α interact in the liver on a genome-wide scale to regulate gene transcription is lacking.

It has been suggested that FXR constitutively sits on FXRREs within target genes as a heterodimer with RXRα. In the absence of bile acids, this FXR-RXRα dimer associates with corepressors to repress gene transcription. Upon binding of bile acids, the FXR-RXRα heterodimer releases corepressors and recruits coactivators and subsequently initiates transcription of FXR target genes (reviewed by Modica et al., 2010). As mentioned above, genome-wide binding studies have shown that FXR displays a high degree of tissue-specific binding (Thomas et al., 2010). This study showed that genes bound by FXR in liver differ vastly from those bound in the intestine

implicating the involvement of other tissue-specific factors responsible for organizing the binding of FXR. Motif analysis of FXR binding sites in liver showed a nuclear receptor half site highly associated with the FXRRE (IR-1) suggesting an orphan nuclear receptor may coordinate FXR binding and function (Chong et al., 2010; Thomas et al., 2010). Further studies revealed liver receptor homolog-1 (LRH-1) is capable of binding to this half site and could be an important nuclear receptor responsible for mediating tissue-specific function of FXR, although this may not be the only orphan nuclear receptor capable of binding to this specific half site (Catalano et al., 2010; Chong et al., 2010; Wilson et al., 1992). On a different note, studies have shown that the orphan nuclear receptor HNF4α is capable of enhancing the liver-specific functions of other type II nuclear receptors. For example, HNF4α has been shown to cooperatively enhance the transcriptional activity of CAR and PXR at the CYP3A4 promoter (Tirona et al., 2003). However, the effects of HNF4α on FXR activity are largely unknown. Therefore, we hypothesize that HNF4α interacts with FXR to enhance FXR function in the liver. In the current study we have compared the genome-wide binding of FXR and HNF4α in mouse livers and characterized cooperation between these two factors on binding to target gene DNA and on regulating gene transcription.

5.3: Results:

5.3.1: ChIP-sequencing.

Genome-wide binding of FXR and HNF4α in mouse livers from previous reports (Schmidt et al., 2010; Thomas et al., 2010) were re-analyzed using MACS. **Figure 13a**

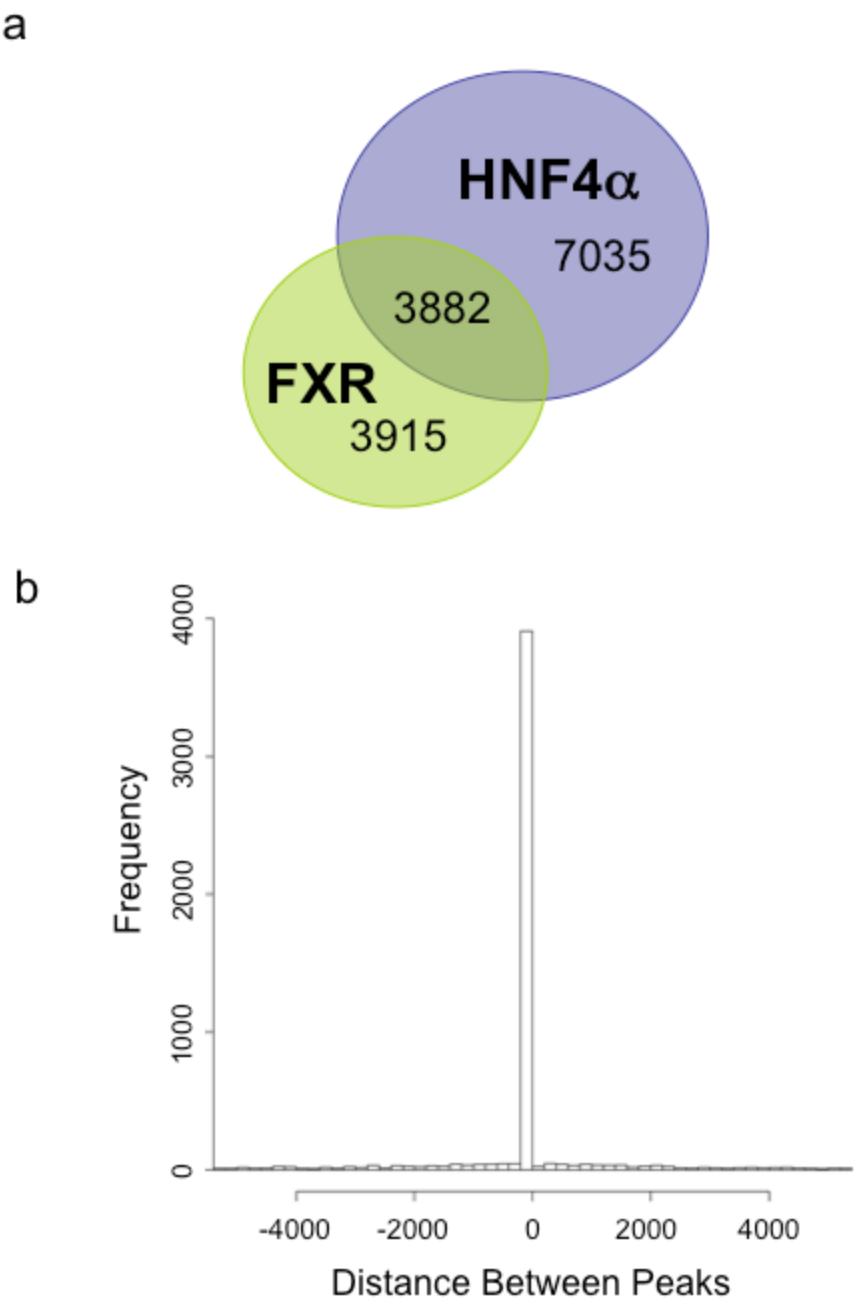
a
HNF4α
7035
3882
FXR
3915
b
Frequency
0
1000
2000
3000
4000
-4000
-2000
0
2000
4000
Distance Between Peaks

Figure 13:

Figure 13: Genome-Wide binding of FXR and HNF4α in mouse liver. a) Venn diagram of total HNF4α and FXR binding sites in mouse livers as revealed by ChIP-seq analysis. There were 10917 total Hnf4α binding sites and 7797 total FXR binding sites mouse liver, of which 3882 binding sites are shared between both factors. b) Histogram of the binding frequency, or number of binding events, of HNF4α (y-axis) in relation to distance from FXR binding site (x-axis) at shared target genes in mouse livers. FXR binding sites are represented by '0.'

shows a venn diagram of total FXR and HNF4α binding sites in mouse livers. There were 10917 total Hnf4α binding sites and 7797 total FXR binding sites mouse liver, of which 3882 binding sites are shared between both factors. These results show 49.7% of total FXR binding sites co-localize with HNF4α. Next, the frequency, or number of binding events (y-axis), of HNF4α binding to shared target genes in relation to distance from FXR binding site (x-axis) was determined. These results showed HNF4α and FXR do not bind to same site, but rather the frequency of HNF4α binding was greatest when bound upstream and in close proximity to FXR (FXR binding site is represented by "0"; **Figure 13b**). Pathway analysis of direct target genes shared by FXR and HNF4α are presented in **Table 10**. Pathways involving compliment and coagulation cascades from this analysis had the highest number of genes targeted by FXR and HNF4α. The list of shared target genes categorized by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps as being a part of complement and coagulation cascades, along with locations of FXR and HNF4α binding sites from gene TSS and relative binding counts of each factor are listed in **Table 11.**

Figure 14a is a histogram of binding of FXR (red) and HNF4α (black) to the Shp gene (*Nr0b2*) in mouse livers, generated by UCSC Genome Browser (http://genome.ucsc.edu; Kent et al., 2002). As can be seen, both the promoter and downstream FXR binding sites within the Shp gene co-localized with those of HNF4α. Sequence analysis of these regions by NUBIScan (Podvinec et al., 2002) showed a classical HNF4α binding site, DR-1, located in the promoter of the Shp gene but not the downstream regulatory region (data not shown), whereas a classical FXR binding site,

Table 10: Pathways enriched by both FXR and HNF4α binding in mouse liver.

Pathway	Genes*	% bound by FXR and HNF4α	P-Value	Bonferroni
Complement and coagulation cascades	17	2.24	1.38E-07	2.25E-05
Drug metabolism	16	2.11	8.33E-07	1.36E-04
PPAR signaling pathway	16	2.11	1.67E-06	2.73E-04
Metabolism of xenobiotics by cytochrome P450	13	1.71	2.97E-05	4.83E-03
Insulin signaling pathway	12	1.58	4.55E-02	0.999
Glycine, serine and threonine metabolism	9	1.18	6.15E-05	9.97E-03
Steroid hormone biosynthesis	9	1.18	7.58E-04	0.116
Adipocytokine signaling pathway	9	1.18	9.80E-03	0.799
Pyruvate metabolism	8	1.05	2.07E-03	0.287
Fatty acid metabolism	8	1.05	3.59E-03	0.443
Drug metabolism	8	1.05	5.19E-03	0.572
Retinol metabolism	8	1.05	3.20E-02	0.995
Cysteine and methionine metabolism	7	0.92	3.10E-03	0.397
Linoleic acid metabolism	7	0.92	1.61E-02	0.929
Biosynthesis of unsaturated fatty acids	6	0.79	6.32E-03	0.644
Starch and sucrose metabolism	6	0.79	2.13E-02	0.970
ABC transporters	6	0.79	4.99E-02	1.000
Selenoamino acid metabolism	5	0.66	1.80E-02	0.948
Alanine, aspartate and glutamate metabolism	5	0.66	4.36E-02	0.999
Primary bile acid biosynthesis	4	0.53	2.74E-02	0.989

Note: * indicates the number of genes bound by FXR and HNF4α in this pathway

IR-1, was previously identified in both of these regions (Li et al., 2010; Thomas et al., 2010)

5.3.2: ChIP-qPCR.

Binding sites that were co-localized by both FXR and HNF4α in mouse livers were analyzed by ChIP-qPCR analysis for binding of HNF4α in WT and FXR KO mouse livers treated with or without GW4064. Binding site locations and counts of FXR and

Table 11: List of FXR and HNF4α shared target genes categorized within complement and coagulation cascades.

Gene Name	Distance from TSS	FXR Counts	HNF4α Counts
Plg	-144, 1992, 6986, 9608	99, 155, 127, 95	46, 39, 31, 33
Fga	-225, -5561	150, 105	40, 53
Cpb2	-9013, 9	52, 34	38, 19
Serpina1e	-4534 , 3769	76, 35	62, 27
Fgg	-345, -4534, 1904, 2607	70, 197, 20, 28	9, 47, 26, 25
F2	-436	129	67
Mbl1	-44, 9558	192, 156	24, 64
Cfh	21	109	21
Kng2	-125, -10481	69, 40	39, 17
Serpine1	-507	115	33
Cfb	-183	217	78
Serpinf2	-66	41	22
C4b	-17142	93	83
C2	-4	68	46
C3	-236, -2276, -2788, -5187	884, 75, 69, 175	46, 15, 10, 55
Proc	-1366	77	53
Kng1	-124, -1946	96. 24	35, 21

HNF4α binding to a few selective shared target genes reveal by ChIP-seq analysis are summarized in **Table 12** and were investigated for HNF4α binding. ChIP-qPCR results showed HNF4α binding to shared target genes, *Apoc3*, *Baat*, *Nr0b2*, and *Sqstm1* was increased in WT mouse livers treated with an FXR agonist, GW4064 (**Figure 14b**, top). Likewise, HNF4α binding to *Apoc3*, *Baat*, and 5' and 3' regulatory regions of *Nr0b2* (Shp) decreased in livers of FXR KO mice (**Figure 14b**, bottom).

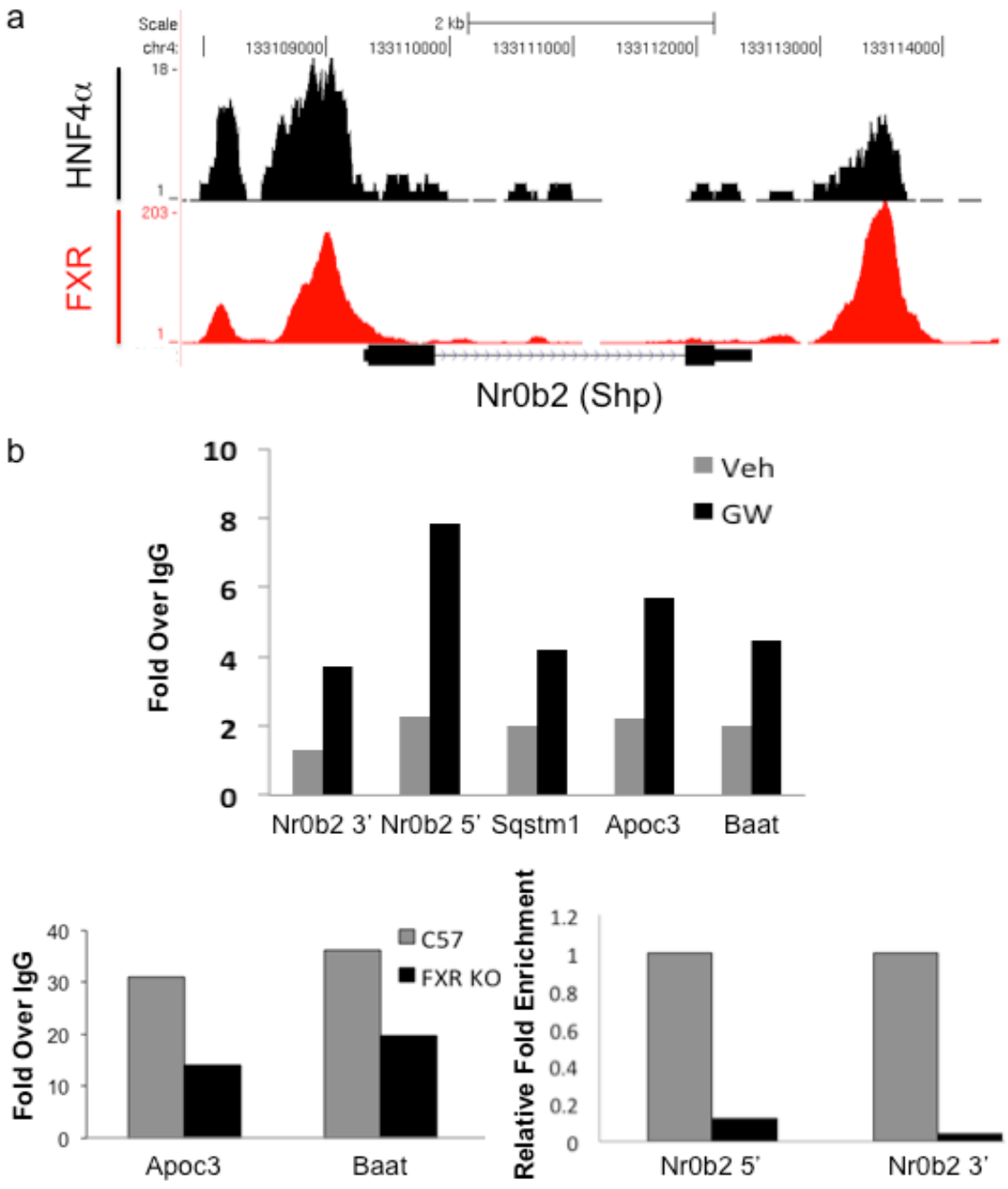
a
Scale
chr4:
2 kb
133109000
133110000
133111000
133112000
133113000
133114000
HNF4α
18
1
FXR
203
1
Nr0b2 (Shp)
b
Fold Over IgG
10
8
6
4
2
0
Veh
GW
Nr0b2 3'
Nr0b2 5'
Sqstm1
Apoc3
Baat
Fold Over IgG
40
30
20
10
0
C57
FXR KO
Apoc3
Baat
Relative Fold Enrichment
1.2
1
0.8
0.6
0.4
0.2
0
Nr0b2 5'
Nr0b2 3'

Figure 14:

Figure 14: Histogram of binding of FXR and HNF4α to *Nr0b2* (Shp) gene and ChIP-qPCR analysis. a) Histogram of the binding of FXR (black) and HNF4α (red) to the *Nr0b2* (Shp) gene in mouse livers as determined by ChIP-seq analysis. Histogram was generated using UCSC genome browser (http://genome.ucsc.edu; Kent et al., 2002). b) Binding of HNF4α to shared target genes, *Nr0b2*, *Sqstm1*, *Apoc3*, and *Baat* in WT mouse livers treated with or without GW4064 (top) or in WT and FXR KO livers (bottom) analyzed by ChIP-qPCR analysis. Results are reported as fold change over IgG negative controls or relative fold enrichment (y-axis).

Table 12: FXR and HNF4α binding sites from ChIP-seq analysis.

Gene Name	Distance to TSS	FXR Counts	HNF4α Counts
Apoc3	-8	94	127
Baat	-107	19	29
Nr0b2	-436	520	193
Nr0b2	4079	771	57
Sqstm1	13061	669	22

5.3.3: Co-IP.

Co-IP assays were carried out on whole cell liver isolates extracted from WT and FXR KO mice fed diets with or without 1% CA. Lysates were immunoprecipitated using an antibody against FXR, and then IP fractions were analyzed by western blot labeling for HNF4α. Using this analysis, a weak FXR-HNF4α protein-protein interaction was detected in WT mice fed control diet (**Figure 15a**). This interaction increased in the livers of mice fed 1% CA diet was nearly undetectable in livers of FXR KO mice (**Figure 15a**).

5.3.4: Luciferase activity.

HNF4α binding to *Nr0b2* promoter and downstream regulatory region was detected by ChIP-seq analysis. These binding sites were analyzed for HNF4α transcriptional activity by using luciferase reporter assays. Transcriptional activity of HNF4α on *Baat* gene promoter has already been characterized (Inoue et al., 2004) and was used as a positive control. These results show that HNF4α significantly increased luciferase activity of *Nr0b2* promoter and the positive control, *Baat*, in a dose dependent

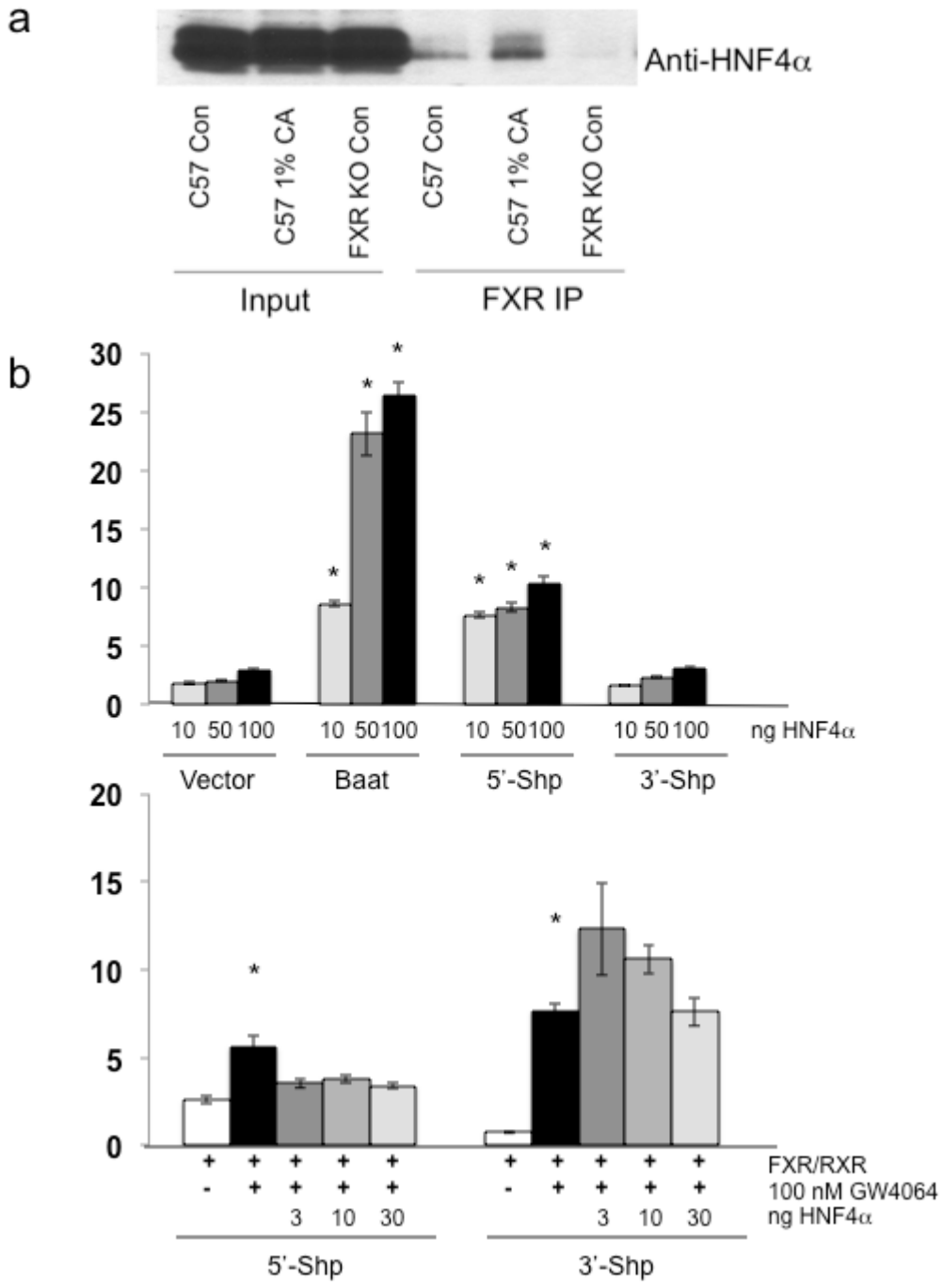
a
Anti-HNF4α
C57 Con
C57 1% CA
FXR KO Con
C57 Con
C57 1% CA
FXR KO Con
Input
FXR IP
b
30
25
20
15
10
5
0
10 50 100
10 50100
10 50100
10 50 100
ng HNF4α
Vector
Baat
5'-Shp
3'-Shp
20
15
10
5
0
FXR/RXR
100 nM GW4064
3 10 30
ng HNF4α
5'-Shp
3'-Shp

Figure 15:

Figure 15: Co-IP of FXR and HNF4α and luciferase assays. a) Co-IP of WT mouse livers fed control diet or 1% CA diet, and FXR KO mouse livers fed control diet. Whole cell liver lysates were prepared and immunoprecipitated using antibody against FXR. Liver lysates (input) and IP fractions were analyzed by western blot analysis using antibody against HNF4α. b) Luciferase expression assays showing the effects of increasing concentrations of mouse HNF4α expression vector on transcriptionally activating regulatory regions in *Baat* gene, 5' region of *Nr0b2* gene, and 3' region of *Nr0b2* gene (top). Bottom panel shows the effects of increasing mouse HNF4α expression vector concentrations on FXR induced transcription of 5' and 3' regions of *Nr0b2* gene after activation of FXR with GW4064. Results are reported as ratio of firefly luciferase activity over renilla luciferase activity (y-axis). '*' $p < 0.05$.

manner, but not the downstream regulatory region of *Nr0b2* (**Figure 15b**, top, * $p < 0.05$).

Next, the effect of HNF4α on the transcriptional activity of FXR at both the promoter and downstream regulatory region of *Nr0b2* was analyzed by luciferase reporter assay. The ability of FXR to transcriptionally regulate the promoter and downstream regulatory region of the Shp gene has already been previously reported (Li et al., 2010). Our results confirm that FXR significantly increases transcriptional activity of both of these sites (**Figure 15b**, bottom, * $p < 0.05$). In addition, at low concentrations (3 ng of expression plasmid) HNF4α increased the transcriptional activity of FXR at the 3' regulatory region of Shp nearly 2-fold, although this increase was not found to be statistically significant. However, HNF4α did not increase transcriptional activity of FXR at the Shp promoter. In fact, it appears there was a slight decrease in transcriptional activity.

5.4: Concluding Remarks:

FXR is a type II adopted nuclear receptor and bile acids are its endogenous ligands (Makishima et al., 1999; Parks et al., 1999). As mentioned, FXR's most well characterized role is in regulating the enterohepatic circulation of bile acids. However, FXR has also been shown to regulate a variety of metabolic processes such as lipid homeostasis, glucose metabolism, insulin sensitivity, and liver and gastrointestinal cancer development (Cariou et al., 2006; Claudel et al., 2003; Kim et al., 2007; Ma et al., 2006; Modica et al., 2008; Staels and Kuipers, 2007; Watanabe et al., 2004; Yang et al., 2007; Zhang et al., 2006). Because of the profound role FXR plays in these processes,

it has become a very promising therapeutic target for the treatment or prevention of cholestasis, hyperlipidemia, fatty liver, type II diabetes, and liver and colon cancer. Genome-wide binding studies have shown that FXR has a very high degree of tissue-specific binding and suggests the involvement of orphan nuclear receptors in regulating FXR function (Chong et al., 2010; Thomas et al., 2010). HNF4α is an orphan nuclear receptor shown to regulate a large array of liver-specific functions. In addition, HNF4α regulates several aspects of bile acid metabolism suggesting possible overlapping function with FXR (Inoue et al., 2004). Furthermore, HNF4α has been shown to cooperatively enhance liver-specific gene transcription of two other type II nuclear receptors, CAR and PXR (Tirona et al., 2003). Therefore, we hypothesize that HNF4α also functions to cooperatively regulate the function of FXR in the liver.

In order to accomplish this, FXR and HNF4α ChIP-seq data in mouse livers were re-analyzed to determine the degree of overlapping binding of these two factors. Even though this analysis was done from different animals, the results showed that nearly 50% of FXR binding sites in liver overlapped with those of HNF4α including co-localization of HNF4α with FXR to both the promoter and downstream regulatory regions of the *Nr0b2* gene that encodes Shp. Of the binding sites that were shared by both factors, the binding frequency of HNF4α was highest when the binding site was located upstream and in close proximity to the FXR binding site. These results suggest that the function of FXR highly correlates with HNF4α function and implicates a possible protein-protein interaction suggested by close proximity of binding. Pathway analysis showed FXR and HNF4α target genes are highly enriched within complement and coagulation cascades and drug metabolism suggesting these may be the pathways

cooperatively regulated by FXR and HNF4α. ChIP-qPCR analysis determined that HNF4α binding to shared target genes, including the promoter and downstream regulatory regions of *Nr0b2*, increased in mouse livers treated with an FXR agonist, GW4064, and decreased in FXR KO mouse livers, suggesting a cooperative and dependent effect of HNF4α binding with FXR. These preliminary results indicate HNF4α binding to these shared target genes is dependent on the presence and function of FXR. Preliminary ChIP-qPCR was also done in livers of WT and hepatocyte-specific HNF4α KO mice and the effects of FXR binding to shared target genes were analyzed. These results showed no difference in FXR binding to *Apoc3* and *Nr0b2* in hepatocyte-specific HNF4α KO livers, and therefore it appears FXR binding to these sites is not dependent on HNF4α binding (data no shown). However, more extensive studies need to be completed in order to experimentally test this hypothesis. For example, binding of FXR in conditional HNF4α KO mice livers treated with an FXR agonist would need to be done to fully evaluate cooperative binding.

Next, co-IP analysis revealed a weak FXR and HNF4α protein-protein interaction in WT mouse livers. This interaction was enhanced in livers of WT mice treated with an FXR agonist, and nearly eliminated in FXR KO mice, confirming an FXR-induced interaction with HNF4α. Finally, luciferase assays showed the HNF4α binding site within the *Nr0b2* promoter, but not the downstream binding site, contains a putative DR-1 and is transcriptionally active. Conversely, HNF4α moderately enhanced transcriptional activity of FXR at the downstream regulatory region of *Nr0b2*, but not the promoter. However, it should be noted that the total effect of HNF4α activity on FXR function may not be fully represented in a luciferase expression assay. This is because HNF4α may

be interacting with FXR to stabilize chromatin microenvironment around the *Nr0b2* gene, which is a process not properly reflected in an artificial expression assays. To illustrate this, a study has shown that the 5' and 3' binding pattern of FXR at the *Nr0b2* gene functions to mediate a head-to-tail chromatin loop around the *Nr0b2* gene (Li et al., 2010). This may be an essential process required for the efficient transcription of the Shp gene in response to FXR activation. ChIP-seq data demonstrated that HNF4α also co-localizes with FXR to 5' and 3' binding sites of the *Nr0b2* gene, therefore, suggesting that HNF4α may be important for mediating FXR induced head-to-tail chromatin loop around the *Nr0b2* gene.

Other studies have implicated that FXR and HNF4α have opposite effects on gene transcription of shared target genes. For example, *Apoc3* is a well characterized HNF4α target gene and HNF4α haploinsufficiency, or decreased hepatic expression of HNF4α, is associated with decreased serum apoC-III levels, therefore, suggesting HNF4α functions to increase transcription of *Apoc3* (Shih et al., 2000; Sladek et al., 1990) . However, FXR has been shown to inhibit the transcription of *Apoc3* by binding to the promoter region of this gene (Claudel et al., 2003). Nevertheless, the transcriptional effect of HNF4α and FXR on shared targets genes on a genome-wide scale was previously unknown. This study suggests that these two factors have an overall cooperative effect on gene transcription of target genes rather than an opposite effect.

In conclusion, we have revealed a high prevalence of co-localization of FXR binding to HNF4α binding in mouse livers, including to 5' and 3' regulatory regions of the Shp (*Nr0b2*) gene. In addition, co-IP and luciferase assays suggest FXR and

HNF4α interact at the protein level and this interaction moderately increases transcriptional activity of FXR at the Shp gene. Therefore, we have determined that FXR and HNF4α interact in the liver to cooperatively regulate liver-specific gene expression in mice.

Chapter 6: DNA Methylation as a Mechanism of FXR Down-regulation in Human Colon Cancer

6.1 Abstract:

High-fat diets increase intestinal exposure to bile acids, thereby, increasing the risk of colon cancer. FXR is a bile acid nuclear receptor critical for minimizing intestinal injury from bile acids. Studies have shown that FXR deficiency in mice promotes colon cancer development, indicating FXR as a tumor suppressor. This study showed that human colon cancer samples and a mouse model for intestinal cancer, APC^{min} mice, had decreased expression of FXR. Likewise, expression of FXR in colon cancer cell lines was inversely related to cancer cells' malignancy. No genetic mutations within the FXR gene or its promoter were found to account for the difference in FXR expression or function in colon cancer cell lines. However, when treated with a clinically used DNMT inhibitor, AZA, human colon cancer cell lines had a marked increase in FXR mRNA, suggesting DNA methylation as a mechanism for FXR silencing in colon cancer. Using bisulfite sequencing and MeDIP analysis, methylation of a FXR promoter CpG island was confirmed in human colon cancer cells and the degree of methylation was inversely correlated to FXR expression. Finally, siRNA knockdown of DNMT1 and 3B in the colon cancer cell line SW620 increased FXR expression, suggesting these enzymes are responsible for aberrant FXR gene methylation. In conclusion, this study has determined that FXR expression is decreased in human colon cancer, further suggesting it as a tumor suppressor, and the mechanism of down-regulation is, in part, due to methylation of FXR promoter.

6.2 Introduction:

Colon cancer is the third most common cancer and the third leading cause of cancer related deaths in the United States. Two major risk factors for colon cancer development are diets high in saturated fat and low in fiber (Armstrong and Doll, 1975). Dietary fats are emulsified by bile acids in the intestine in order for proper digestion and absorption. However, high fat diets leads to an elevated intestinal bile acid load, and low dietary fiber increases the gastrointestinal transit time (Correa, 1981; Willett et al., 1990). Together these factors increase both level and time of bile acid exposure.

In the distal ileum and colon, primary bile acids are converted to secondary bile acids by intestinal microflora (Hofmann, 1999). Secondary bile acids, DCA and LCA, are considered to be more cytotoxic compared to primary bile acids, CA and CDCA, due to their detergent-like properties and higher hydrophobicity (Hofmann, 1999). DCA and LCA can induce colonic epithelium cytotoxicity through oxidative stress and promote cell proliferation, and therefore, are linked to increased colon carcinogenesis (Lechner et al., 2002). In fact, it has been shown that patients with colorectal cancer have elevated levels of secondary bile acids in their feces (Bianchini et al., 1989; Hill et al., 1975; Reddy et al., 1978).

FXR is a ligand-activated TF belonging to the nuclear receptor superfamily. FXR is highly expressed in liver and intestine and bile acids are its endogenous ligands (Forman et al., 1995; Parks et al., 1999). FXR critically regulates every aspect of the enterohepatic circulation of bile acids (Kok et al., 2003; Sinal et al., 2000). FXR functions to regulate the synthesis, transport, intestinal re-absorption, and free intracellular concentration of bile acids to prevent their accumulation to cytotoxic levels

(Inagaki et al., 2005; Okuwaki et al., 2007; Sinal et al., 2000; Tu et al., 2000; Zollner et al., 2006). Furthermore, FXR has been shown to attenuate the development of intestinal tumors in mouse models of colon cancer (Maran et al., 2009; Modica et al., 2008). This implicates FXR as a tumor suppressor for the development of colon cancer. However, the exact mechanisms of FXR induced tumor suppression are unknown. One suggested mechanism is through the protection of intestinal epithelium from bile acid toxicity by up-regulating intracellular bile acid binding proteins and efflux transporters while down-regulating bile acid influx transporters and *de novo* synthesis of bile acids. However, FXR appears to have anti-tumorigenic functions independent of its regulation of bile acid homeostasis. For example, FXR deficiency increases susceptibility to colon cancer development by increasing epithelial permeability to bacteria and promoting WNT/β-catenin signaling as a result of TNFα released from infiltrating macrophages (Inagaki et al., 2006; Modica et al., 2008).

FXR expression is inversely related to the malignancy of colon cancer cell lines, implicating colon cancer cells have developed a mechanism to selectively down-regulate FXR (De Gottardi et al., 2004). Although polymorphisms within the FXR gene have been identified and associated with decreased function in intrahepatic cholestasis of pregnancy (ICP; Marzolini et al., 2007), no clinically known mutations exist within the FXR gene to explain decreased FXR expression or activity in human colon cancer. Therefore, it has been proposed that FXR expression is regulated by epigenetic mechanisms. DNA methylation is a common epigenetic mechanism involved in gene silencing. Cancer has been associated with an overall hypomethylation of genomic DNA, but hypermethylation of CpG islands located in promoter regions of tumor suppressor

genes (Baylin et al., 1998). A putative CpG island located just upstream of FXR gene (*NR1H4*) TSS was identified and proposed to be the site of DNA methylation responsible for FXR gene silencing in human colon cancer. This study was designed to determine the expression profile of FXR in human colon cancer and mouse intestinal cancer model and to test whether DNA methylation of the FXR promoter is responsible for FXR down-regulation in colon cancer.

6.3 Results:

6.3.1 FXR expression in intestinal carcinogen mouse model.

APC^{min} mice are a common intestinal carcinogen animal model (Su et al., 1992). Nearly 80% of all human colon cancers, inherited or sporadic, arise due to a mutation in the APC gene (Goss and Groden, 2000).This mouse model was used to investigate the expression levels of FXR in intestine. There are two isoforms of FXR, α and β, expressed in mouse ileum and colon (Zhang et al., 2003). Therefore, the expression of both of these isoforms was measured by real-time qPCR. These results show that mRNA levels of both isoforms of FXR were significantly decreased in the ileum and colon of 1-year-old APC^{min} mice, with a 3- and 11-fold decrease of isoforms α and β in ileum, and roughly 2.5-fold decrease of both isoforms in colon (**Figure 16a**, * $p < 0.05$)**.** Therefore, in at least one mouse model of intestinal cancer, APC^{min} mice, FXR is down-regulated.

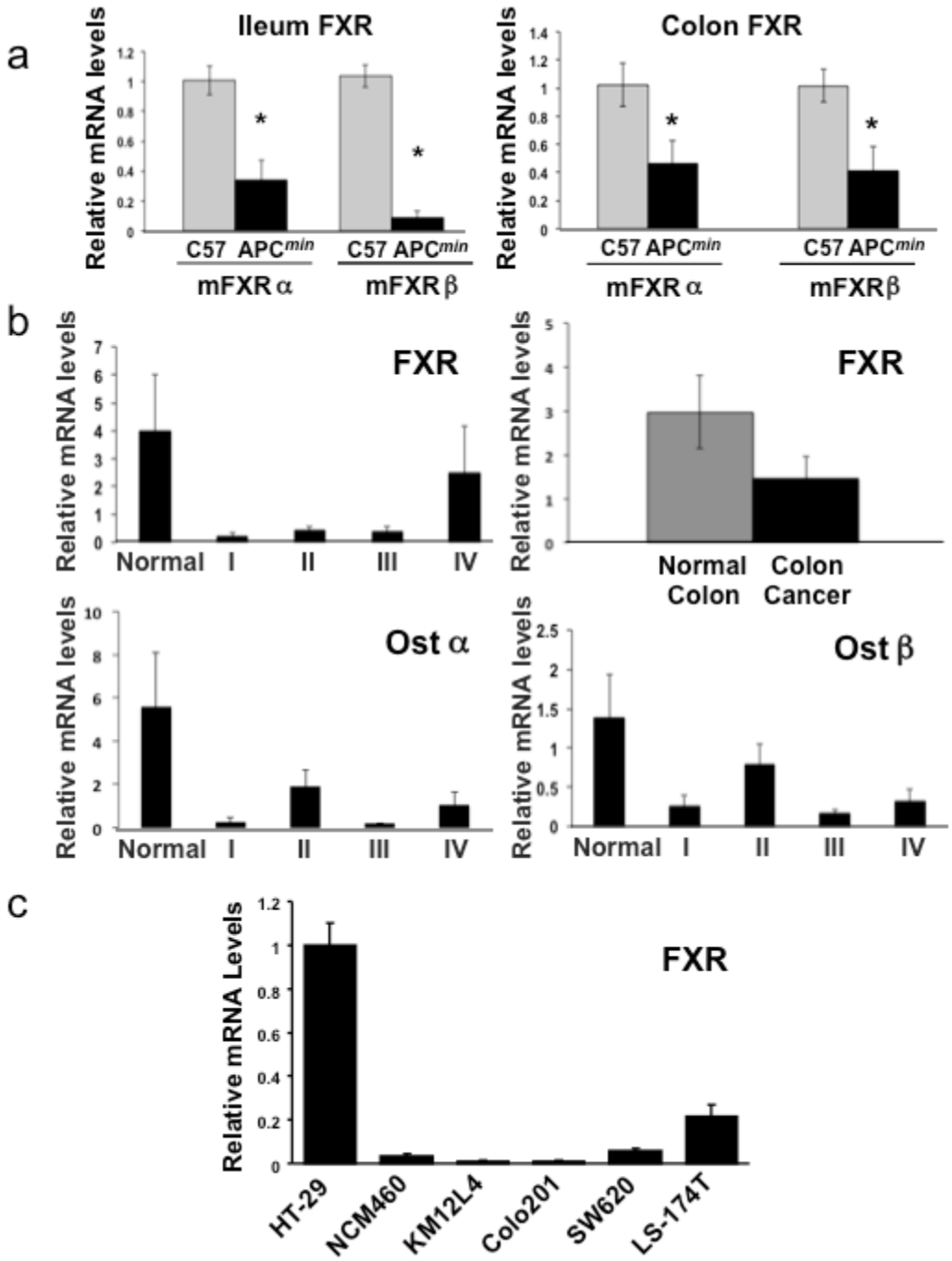
a
Ileum FXR
Relative mRNA levels
C57 APCmin
C57 APCmin
mFXR α
mFXR β
Colon FXR
Relative mRNA levels
C57 APCmin
C57 APCmin
mFXR α
mFXR β
b
FXR
Relative mRNA levels
Normal I II III IV
FXR
Relative mRNA levels
Normal Colon
Colon Cancer
Ost α
Relative mRNA levels
Normal I II III IV
Ost β
Relative mRNA levels
Normal I II III IV
c
FXR
Relative mRNA Levels
HT-29
NCM460
KM12L4
Colo201
SW620
LS-174T

Figure 16:

Figure 16: Relative expression of FXR and FXR target genes in APC^{min} mice, human colon cancer samples, and human colon cancer cell lines. a) Relative mRNA levels of FXRα and FXRβ in ileum (left) and colon (right) of APC^{min} mice. b) Relative mRNA levels of FXR in two sample sets of human colon cancer samples (top), and relative mRNA levels of OSTα and OSTβ first sample set (bottom). c) Relative mRNA levels of FXR in non-metastatic colon cancer cell line HT-29 versus transformed or malignant/metastatic colon cancer cell lines, NCM460, KM12L4, Colo201, SW620, LS-174T . '*' $p<0.05$.

6.3.2 FXR expression in human colon cancers.

In **Figure 16b,** mRNA levels of FXR and FXR target genes OSTα and OSTβ, were down-regulated in all stages of colon cancer development. Decreased expression of these genes was not found to be statistically significant in this sample set, likely due to the low sample number included in this analysis.

Messenger RNA levels of FXR were also measured in different colon cancer cell lines (**Figure 16c**). These colon cancer cell lines differ in their degree of malignancy. HT-29 cells have been previously characterized as being a more differentiated colon cancer cell line, where as KM12L4, Colo201, SW620, and LS-174T cells have been described as more malignant colon cancer cell lines (De Gottardi et al., 2004; Leibovitz et al., 1976; Morikawa et al., 1988; Semple et al., 1978; Tom et al., 1976; Trainer et al., 1988). NCM460 cells are a normal colonic epithelial cell line described as being non-tumorigenic (Moyer et al., 1996). However, subcultures of cells have shown a transformed phenotype, abnormal karyotype and may be tumorigenic according to product information (Incell, San Antonio, TX). The results from this study showed that the expression profile of FXR differed in different colon cancer cell lines with mRNA levels of FXR inversely related to the malignancy of the colon cancer cell line. In order to determine if DNA mutation of the FXR gene (*NR1H4*) is responsible for decreased expression levels, the entire FXR gene and 5 kb upstream promoter were sequenced in Caco-2, HT-29, and SW620 cells, which have different basal expression levels of FXR. No mutations that could account for the difference in FXR expression levels in these different colon cancer cell lines were detected (data no shown).

6.3.3 Azacytidine treatment.

Because no mutation was found in the FXR gene coding region in human colon cancer cells, epigenetic modification of the FXR gene are hypothesized to be responsible for the decreased FXR expression in colon cancer. In order to determine if DNA methylation was responsible for suppression of FXR gene expression, a panel of cDNAs prepared from 11 different colon cancer cell lines treated with a DNMT inhibitor, AZA, was used to measure mRNA levels of FXR. This preliminary screen revealed 6 out of 11 of these colon cancer cell lines showed a 1.7 to 233 fold increase in FXR expression after AZA treatment (**Table 13**). To confirm this, colon cancer cell lines HT-29 and SW620, which have different basal expression of FXR and different responses to AZA as revealed by the original screen, were treated with AZA and then mRNA levels of FXR and COL1A2 (positive control gene known to be methylated in colon cancers) were measured (**Figure 17a**). As mentioned, these colon cancer cell lines differ in their relative malignancy and differentiation, where HT-29 cells are considered more differentiated cells and SW620 cells more malignant (De Gottardi et al., 2004). This

Table 13: FXR expression in colon cancer cell lines after AZA treatment.

Cell Line	Fold Chg After AZA Treatment (2 μg/mL)
NCM460	2.95
CCD841	1.66
SW480	0.53
SW620	49.92
KM12C	1.26
KM12L4	233.31
HT-29	1.20
HCT116	0.78
Colo 201	15.04
Colo 205	0.50
LS-174T	5.36

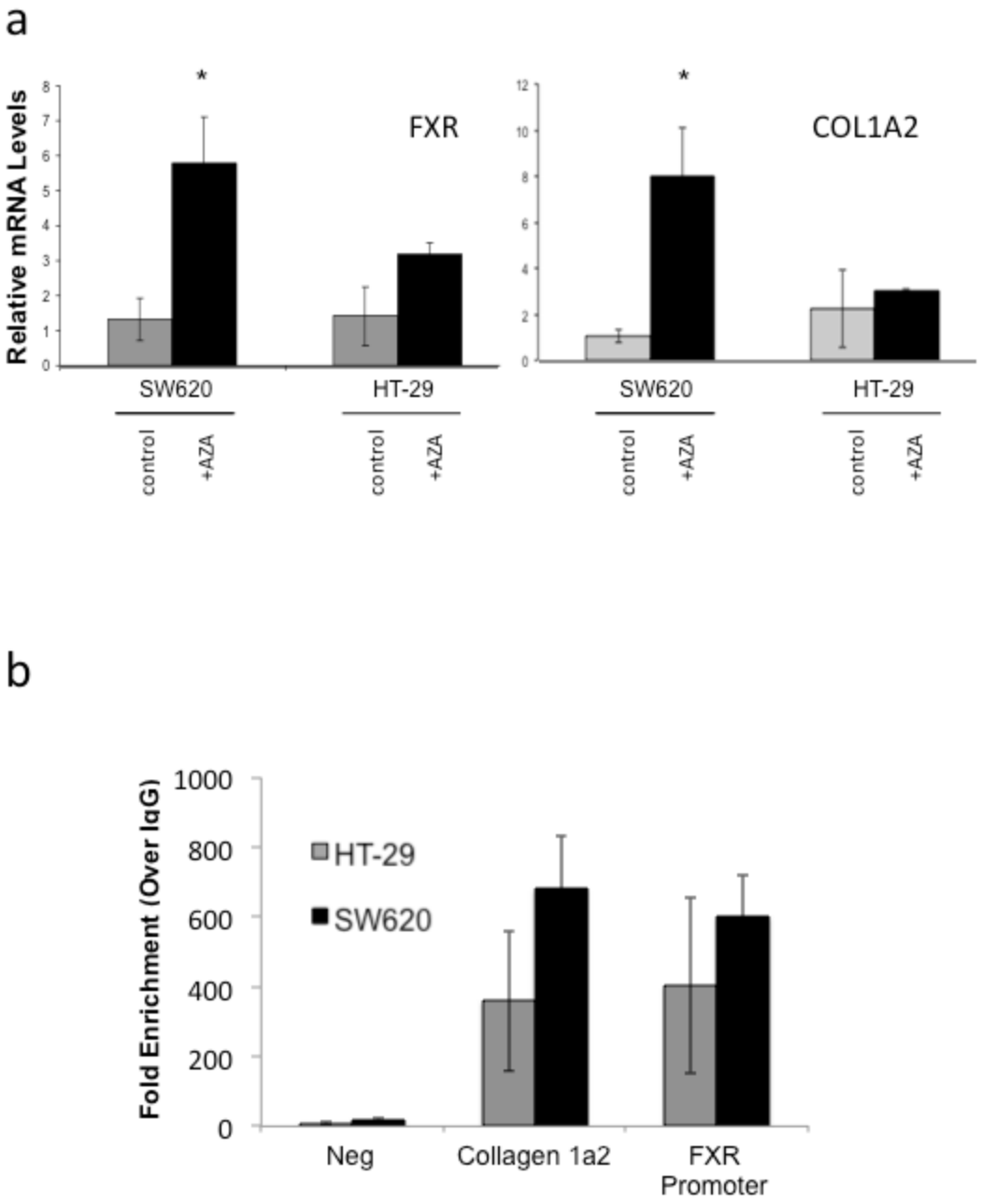
a
Relative mRNA Levels
FXR
COL1A2
*
*
SW620
HT-29
SW620
HT-29
control
+AZA
control
+AZA
control
+AZA
control
+AZA
b
Fold Enrichment (Over IgG)
1000
800
600
400
200
0
HT-29
SW620
Neg
Collagen 1a2
FXR Promoter

Figure 17:

Figure 17: Relative expression of FXR in human colon Cancer cell lines treated with a DNMT inhibitor and MeDIP analysis of FXR promoter CpG island. a) Relative mRNA levels of FXR in HT-29 and SW620 cells treated with 2ug/mL of AZA for three days. b) Immunoprecipitation of genomic DNA from HT-29 and SW620 cells with antibody against 5-mC followed by qPCR analysis of immunoprecipitated DNA. Methylation of a CpG island located near COL1A2 TSS was analyzed as positive control, and an unmethylated genomic region was analyzed for the negative control. To determine the immunoprecipitation of predicted FXR promoter CpG island, primers were designed specifically to amplify this CpG island region. '*' $p<0.05$.

experiment showed that AZA treatment significantly increased mRNA levels of both FXR and COL1A2 in SW620 cells 6- and 8- fold (* $p < 0.05$), but not in HT-29 cells. Therefore, DNA methylation seems to be responsible for FXR gene suppression in SW620 cells, but not in HT-29 cells. Consequently, DNA methylation of the FXR gene was further investigated. In order to further determine whether DNA methylation of the FXR gene is responsible for suppression of FXR gene expression in human colon cancer, the FXR gene and 5 kb promoter sequence was analyzed for the presence of predicted CpG islands using MethPrimer (Li and Dahiya, 2002). As mentioned, 6 predicted CpG islands were identified by this analysis (**Figure 6a**). However, only methylation of the CpG island located in the promoter of FXR was analyzed in detail because this is typically the location of methylated CpG islands used for suppression of gene expression (Baylin et al., 1998). There are 11 predicted CpG sites located within this CpG island, as shown as a dashed line with the CpG sites are shown as black circles. The predicted sequence of this CpG island is shown in **Figure 6b**, with the dinucleotide CG underlined in the sequence.

6.3.4 MeDIP analysis.

Methylation of the FXR promoter CpG island was first analyzed by MeDIP analysis. This assay uses IP of genomic DNA using an antibody against 5-mC followed by qPCR analysis of IP DNA fragments. The MeDIP assay was done on genomic DNA isolated from HT-29 and SW620 cells. Identification of a methylated CpG island was first confirmed near the TSS of COL1A2 gene, which has been previously characterized in colon cancer cell lines (Sengupta et al., 2003). Likewise, a housekeeping gene region

found to not be methylated was used as a negative control for this assay (Sorensen et al., 2010). The results showed that, similar to the positive control with detectable levels of methylation of a CpG island located within a regulatory region of COL1A2, the CpG island within the FXR gene promoter had detectable methylation in both HT-29 and SW620 cells, around 400 and 600 fold over IgG, when compared to the non-methylated region (negative control), which was around 9 and 20 fold over IgG (**Figure 17b**). Likewise, there seemed to be a slightly higher trend in COL1A2 and FXR promoter CpG island methylation in the SW620 cells, around 1.5 to 2 fold higher, than in HT-29 cells, although this is not statistically significant.

6.3.5 Bisulfite treatment.

MeDIP analysis is commonly used for the detection of methylated CpG islands but is insufficient in quantifying levels of methylation of these CpG islands. Therefore, to further characterize the degree and pattern of methylation of the confirmed CpG island within the FXR promoter, bisulfite sequencing analysis of this island was performed. In order to correlate level of CpG island methylation to the expression of FXR in colon cancer cell lines, HT-29, Caco-2, and SW620 cells were grown for 3, 7, 14, and 21 days and total RNA and genomic DNA were extracted. In all three cell lines, mRNA levels of FXR increased as cells grew to confluence, and reached maximal levels at day 14 (**Figure 18a**). At 21 days, FXR expression in HT-29, Caco-2, and SW620 either slightly decreased or leveled off. Genomic DNA was also collected from cells grown for 7, 14, and 21 days were used for bisulfite sequence analysis. The results are reported as the

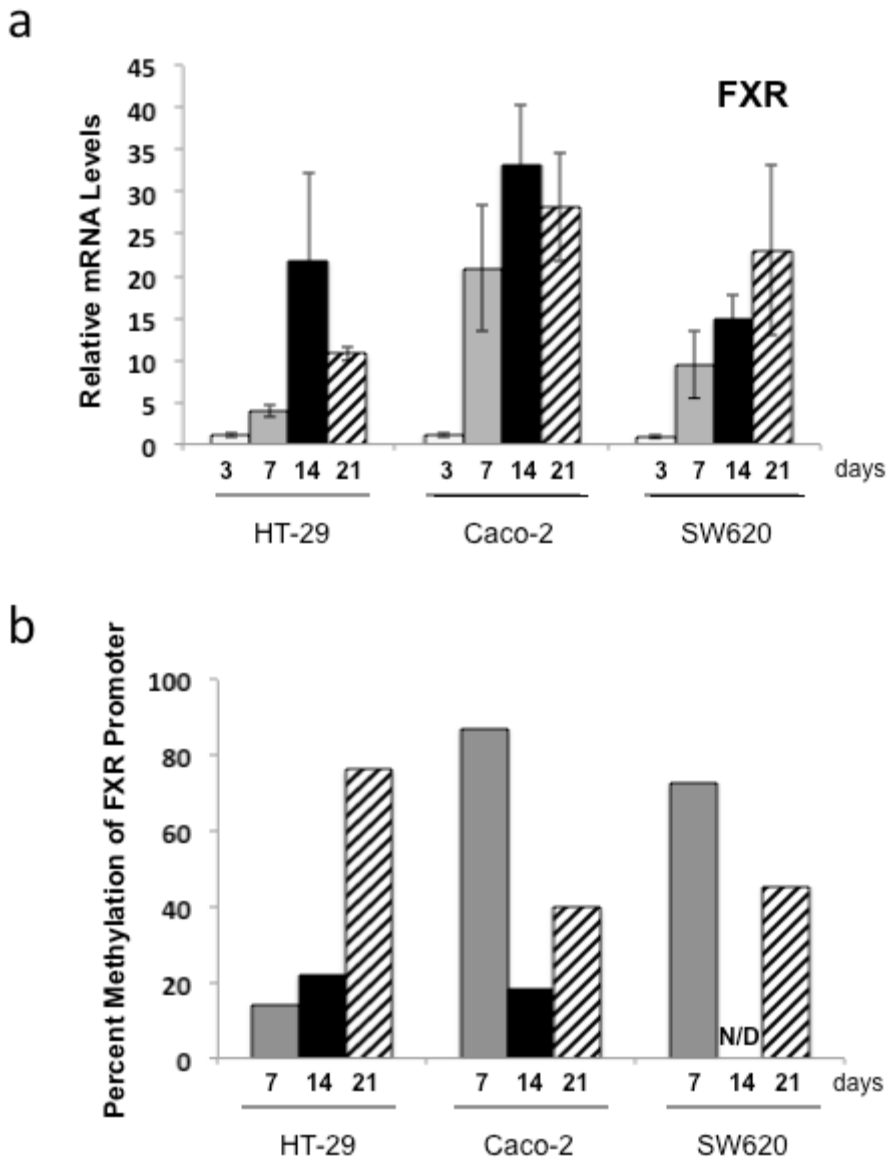
a
FXR
Relative mRNA Levels
45
40
35
30
25
20
15
10
5
0
3 7 14 21
3 7 14 21
3 7 14 21
days
HT-29
Caco-2
SW620
b
Percent Methylation of FXR Promoter
100
80
60
40
20
0
N/D
7 14 21
7 14 21
7 14 21
days
HT-29
Caco-2
SW620

Figure 18:

Figure 18: Relative expression of FXR and bisulfite sequencing analysis of FXR promoter CpG island in colon cancer cell lines. a) Relative mRNA levels of FXR in HT-29, CaCO2, and SW620 cells grown 3, 7, 14, and 21 days. b) Bisulfite sequencing results of FXR promoter CpG island in HT-29, Caco-2, and SW620 cells grown 7, 14, and 21 days. Results are reported as the percentage of CpG island methylation, or number of CpG sites methylated out of 11 total predicted CpG sites (N/D indicates non-detectable methylation).

percentage of CpG island methylation in the FXR promoter (**Figure 18b**). Eight clones, generated from bisulfite conversion and PCR amplification, from each cell line grown for 7, 14, and 21 days were sequenced and the number of methylated CpG sites out of 11 total predicted CpG sites were calculated. Using this analysis, we were unable to detect any specific pattern of methylation in this CpG island which correlated with FXR expression. However, there was an inverse correlation of the percentage of FXR promoter methylation with FXR expression in Caco-2 and SW620, and only a slight correlation in HT-29. Specifically, Caco-2 and SW620 cells grown for 7 days, which had relatively lower levels of FXR mRNA, had increased percentage of FXR promoter CpG island methylation, around 87% and 72%. Conversely, at 14 days of growth, Caco-2 and SW620 cells had decreased CpG island methylation of FXR promoter at 18% and 0%, which correlates to maximal FXR mRNA levels in these cells. HT-29 cells maintained low levels of CpG island methylation, 22 and 14%, for cells grown both 7 and 14 days, even though FXR mRNA levels changed from relatively lower levels at 7 days to maximal levels at 14 days. At 21 days of growth, mRNA levels of FXR expression seemed to decrease or level off in HT-29, Caco-2, and SW620 cells. This was associated with an increase or mixed percentage of methylation of FXR promoter CpG island, at around 76%, 39%, and 45% for HT-29, Caco-2, and SW620 cells. Nevertheless, it appears that DNA methylation of FXR promoter CpG islands is associated with the difference in FXR expression seen in Caco-2 and SW620 cells, although this correlation was not as strong in HT-29 cells.

6.3.6 SiRNA knockdown.

SiRNA knockdown of DNMT 1 and 3B was done to determine the molecular machinery responsible for FXR down-regulation. SW620 cells have lower basal levels of FXR expression when compared to HT-29 cells, and responded to AZA treatment, therefore, these cells were used for DNMT siRNA knockdown. SW620 cells were transfected with siRNA designed to knockdown DNMT 1 or 3B, and RNA was extracted and analyzed for DNMT 1, DNMT 3B, FXR, and COL1A2 mRNA levels. Messenger RNA levels of these genes were normalized to mRNA levels of GAPDH. This study showed that knockdown of DNMT 1 and 3B, by nearly 80% (**Figure 19a**, * $p < 0.05$) were both sufficient to increase mRNA levels of FXR 6.7 and 7.3 fold, and positive control gene COL1A2 around 7 and 6.6 fold (**Figure 19b**), although these values were not found to be statistically significant over non-targeting siRNA transfection controls. However, the increase in FXR and COL1A2 mRNA after DNMT 1 and 3B siRNA knockdown reach statistical significance or near significant levels when compared to the non-transfected control levels (statistical significance indicated by parenthesis, p-value < 0.05).

6.4 Concluding Remarks:

Colon cancer is a significant health burden for the United States and its impact worldwide is increasing due to the adoption of western diet in developing countries. The high fat and low fiber components of the western diet have been highly associated with increased risk of colon cancer development (Armstrong and Doll, 1975; Correa, 1981). Bile acids are amphipathic molecules essential for the proper digestion and absorption

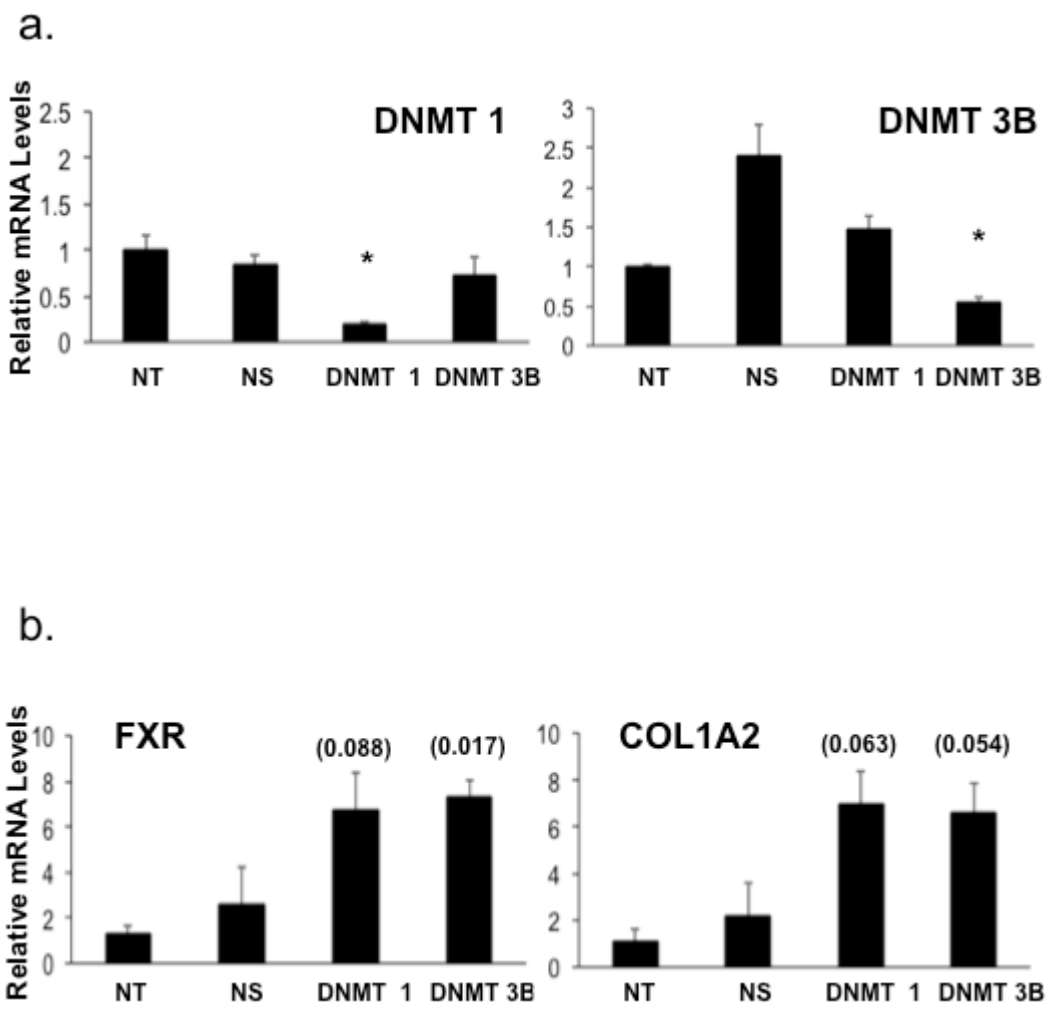
a.
Relative mRNA Levels
DNMT 1
DNMT 3B
NT
NS
DNMT 1
DNMT 3B
b.
FXR
(0.088)
(0.017)
COL1A2
(0.063)
(0.054)

Figure 19:

Figure 19: SiRNA knockdown of DNMT 1 and 3B. SW620 cells were transiently transfected with siRNA designed to knockdown DNMT 1, 3B, or non-specific oligonucleotides. Knockdown was confirmed by real-time qPCR. a) Messenger RNA levels of DNMT 1 (left) and DNMT 3B (right) was nearly 80% that of the non-specific siRNA control. b) Relative mRNA levels of FXR (left) and COL1A2 (right) in SW620 cells after siRNA knockdown of DNMT 1 and 3B. COL1A2 has been previously shown to be methylated in SW620 cells and, therefore was used as a positive control (Sengupta et al., 2003). '*' $p < 0.05$. Values in parenthesis indicate near statistical increase of FXR and COL1A2 mRNA compared to non-transfected control cells.

of fats, and increased dietary consumption of fats correlates to an increased bile acid load in the intestine (Cummings et al., 1978). Although bile acids are essential for survival, accumulation of bile acids to high concentrations has been linked to increased colon tumorigenesis (Bernstein et al., 2011; Lechner et al., 2002; Pai et al., 2004).

FXR is an adopted nuclear receptor responsible for intricately regulating free bile acid levels in both the liver and intestine and has been suggested to serve as a potential tumor suppressor for the development of colon cancer. In fact, mice deficient in FXR show increased colonic tumorigenesis (Maran et al., 2009; Modica et al., 2008). However, studies have shown that FXR anti-tumorigenic effects are at least partially due to bile acid independent mechanisms, namely by regulating intestinal integrity and inflammation (Inagaki et al., 2006; Modica et al., 2008). Identifying FXR as a potential tumor suppressor suggests FXR as a possible target for the prevention and/or treatment of human colon cancer. However, more information on FXR's role in the development in human colon cancer is needed.

In this study, we have revealed that FXR is significantly down-regulated in a common mouse model of intestinal carcinogenesis, APC^{min} mice, which is consistent with previous studies (Modica et al., 2008). In addition, FXR is highly down-regulated in all stages of human colon cancer. Low sample number may have caused this decrease to lack statistical significance. However, expression of FXR target genes, OSTα and β, were also down-regulated suggesting that the suppression of FXR in these samples is indeed biologically significant. In addition, our findings are consistent with previous reports (De Gottardi et al., 2004). We also showed that human colon cancer cell lines have different expression profiles of FXR and there seems to be an inverse correlation

between FXR expression and malignancy of the colon cancer cell line. Sequencing of the FXR gene and promoter regions in colon cancer cell lines showing differences in FXR expression revealed no obvious mutations or SNPs that could account for the expression difference. Therefore, we hypothesized epigenetic modification(s) of the FXR gene being responsible for the difference in FXR expression seen in these colon cancer cell lines.

In an initial screen, treatment of colon cancer cell lines with a clinically used DNMT inhibitor, AZA, resulted in an increase in FXR mRNA levels in 6 out of 11 cell lines tested. In confirmation of this, treatment of malignant colon cancer cell line, SW620 cells but not HT-29 cells, with AZA caused a corresponding increase in FXR mRNA levels suggesting DNA methylation of the FXR gene as the epigenetic mechanism involved in FXR gene silencing in human colon cancer. Sequence analysis revealed a putative CpG island located upstream of the FXR (*NR1H4*) gene which could serve as a potential site for DNA methylation responsible for FXR gene silencing. MeDIP analysis confirmed methylation of this CpG island in human colon cancer cell lines, HT-29 and SW620, and the more malignant cell line, SW620 cells, showed slightly higher levels of CpG island methylation. Bisulfite sequencing analysis was done to more specifically determine the methylation pattern of this CpG island and to associate the degree of FXR promoter methylation with decreased expression of FXR. We found no obvious methylation pattern within this particular island, meaning no specific CpG sites within this island appeared to be of particular importance for FXR gene suppression. However, an inverse correlation of the percentage of FXR promoter CpG island methylation with FXR expression in colon cancer cell lines Caco-2 and

SW620 was seen, but only a weak correlation detected in HT-29 cells. This could suggest that DNA methylation of the FXR promoter decreases FXR expression in some colon cancer cell lines, but not others. One observation important to point out is the lack of direct correlation between FXR promoter methylation to FXR mRNA levels across the three different colon cancer cell lines, demonstrating the involvement of other epigenetic mechanisms in regulating the expression of FXR. However, siRNA knockdown of DNMT 1 and 3B both resulted in an overall increase in FXR mRNA levels in SW620 cell, further validating the role of DNA methylation as a mechanism of FXR silencing. Although these results were not found to be statistically significant, increased expression of the positive control gene, COL1A2, and validation by a repeat experiment (data not shown), confirms the biological significance of this effect. These results, thus, suggest that DNMT 1 and 3B are both capable of methylating FXR promoter, resulting in silencing of this gene.

Clearly, DNA methylation of the FXR promoter is only partially responsible for regulating FXR expression in human colon cancer. Therefore, other mechanisms are likely also responsible. For example, over expression of histone deacetylases and mRNA instability have also been linked to silencing of tumor suppressor genes (Ellis et al., 2009; Guil and Esteller, 2009; Nelson and Weiss, 2008; Zhu et al., 2004), and these mechanisms may be involved in silencing FXR. Furthermore, little is known about TFs that regulate the expression of FXR. Therefore, it is possible that another factor regulates intestinal expression of FXR which is, itself, silenced by DNA methylation. **Figure 20** illustrates our prediction of FXR silencing in human colon cancer. This study has demonstrated that DNA methylation of FXR promoter is, at least partially,

responsible for FXR silencing in colon cancer. These results correlated a high FXR promoter methylation to low expression of FXR, low promoter methylation to higher levels of FXR expression, and mixed promoter methylation partial FXR gene expression (**Figure 20a**). Conversely, our data also predict the presence of an unknown factor(s), which positively regulates intestinal FXR expression under normal physiological processes, but is silenced in human colon cancer by promoter methylation (**Figure 20b**). Consequently, inhibiting DNA methylation reverses the silencing of this gene, and therefore, restores basal expression of FXR. This scenario is consistent with our results showing increased FXR expression in colon cancer cell lines after inhibition of DNMT activity. Lastly, FXR has also been shown to be down-regulated in the acute phase response initiated by LPS and TNFα signaling (Savkur et al., 2005). Therefore, an alternative inflammatory mechanism may also be responsible for FXR down-regulation in human colon cancer.

In conclusion, we confirmed FXR is down-regulated in both human colon cancer and mouse models of intestinal cancer. We have shown that down-regulation of FXR in human colon cancer is in part due to DNA methylation of the FXR promoter. Inhibition of FXR gene silencing, by reversing promoter methylation or other mechanisms, could restore basal expression of FXR in human colon cancer and, therefore, slow the progression of colon cancer development. This mechanism could potentially be exploited as a treatment and/or prevention option for human colon cancer and, therefore, needs to be further investigated.

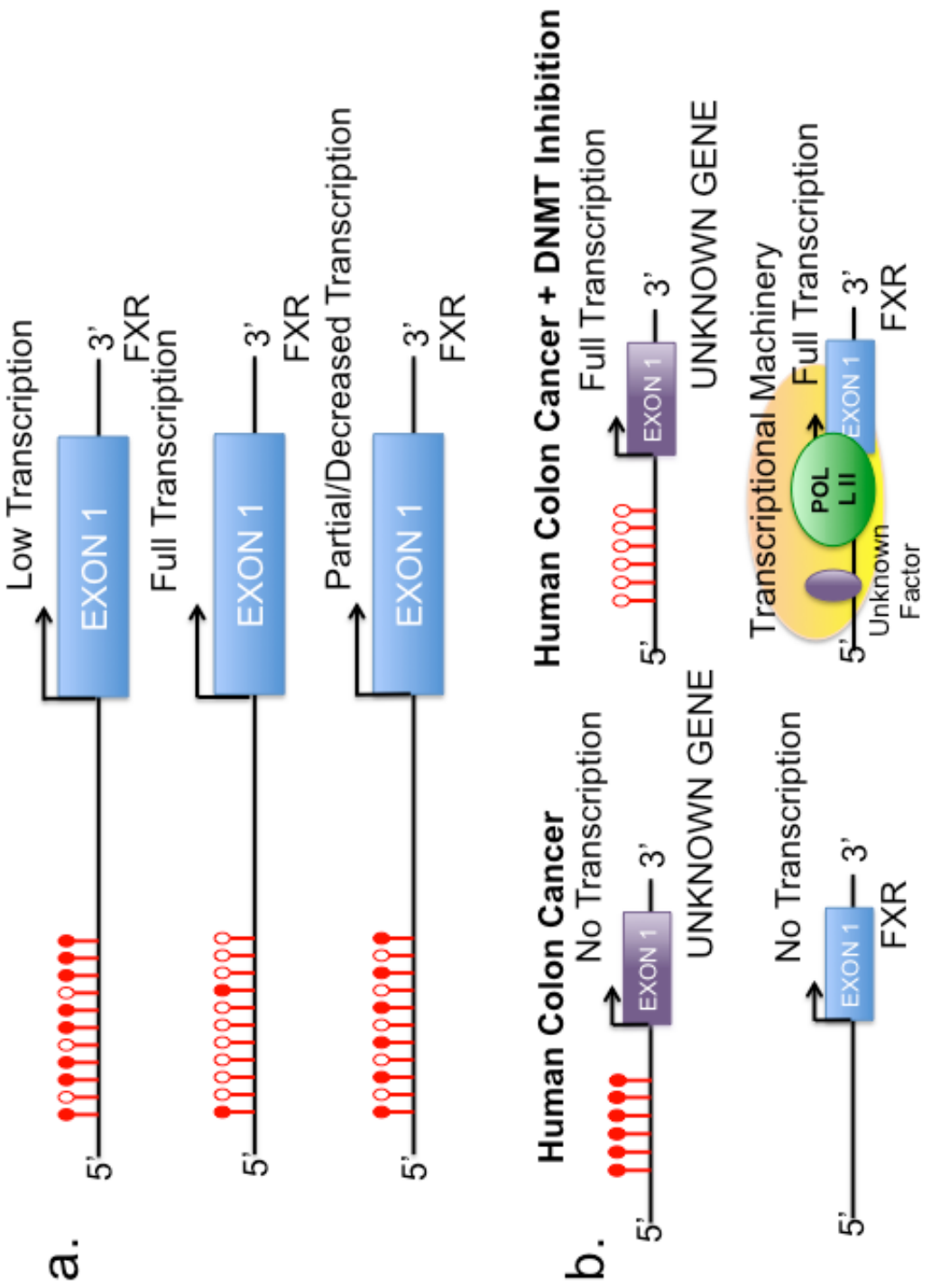
a.
Low Transcription
EXON 1
5'
3'
FXR
Full Transcription
EXON 1
5'
3'
FXR
Partial/Decreased Transcription
EXON 1
5'
3'
FXR
b.
Human Colon Cancer
No Transcription
EXON 1
5'
3'
UNKNOWN GENE
Human Colon Cancer + DNMT Inhibition
Full Transcription
EXON 1
5'
3'
UNKNOWN GENE
No Transcription
EXON 1
5'
3'
FXR
Transcriptional Machinery
Full Transcription
POL LII
Unknown Factor
EXON 1
5'
3'
FXR

Figure 20:

Figure 20: Schematic of FXR silencing in human colon cancer. a) FXR promoter methylation is in part responsible for silencing FXR expression in human colon cancer. Our results revealed that high percentage of promoter methylation was associated with lower levels of FXR expression in colon cancer cell lines (top). Conversely, low percentages of FXR promoter methylation was associated with elevated levels of FXR expression (middle). Lastly, in human colon cancer cell lines grown for 21 days, FXR expression either leveled off or dropped, and this was associated with a mixed or increased level of FXR promoter methylation (bottom). b) There is likely an unknown mediator of FXR transcription in the intestines. This diagram shows the unknown factor with a hypermethylated promoter resulting in the silencing of this gene in human colon cancer. The expression of this gene is necessary for the proper expression of FXR, and therefore, FXR expression is decreased in response to the silencing of this unknown factor. Conversely, inhibition of DNA methylation reverses the silencing of this factor, and restores its expression. There are now sufficient levels of this unknown factor is available to bind to the FXR promoter, or other regulatory regions, recruit transcriptional machinery, and initiate transcription of FXR, therefore, restoring FXR expression to basal levels.

Chapter 7: Discussion and Conclusions

7.1 General Discussion:

Viewing FXR as a potential therapeutic target for the treatment of several metabolic disorders drives the desire to better understand the physiological roles of FXR. Within the last two decades, research on FXR has exploded bringing to light the real potential for using FXR modulators to prevent and treat cholestasis, hyperlipidemia, type II diabetes, fatty liver, and liver and colon cancer. However, as is the case with therapeutically targeting other nuclear receptors, systemic activation of FXR may be associated with adverse health effects. Therefore, much attention is being paid to developing selective FXR modulators that function to enhance beneficial FXR activity, while minimizing undesired effects of FXR. This has guided the field towards understanding the epigenetic mechanisms responsible for regulating gene-specific, cell-specific, and tissue-specific functions of FXR.

Epigenetics are tightly regulated molecular mechanisms capable of altering expression of a gene without modifying the underlying DNA sequence. Epigenetic mechanisms are thought to be involved in many cellular functions such as development, tissue-specific expression of genes, senescence, and development of diseases, such as cancer. More recently, epigenetics has been shown to be responsible for regulating nuclear receptor-mediated transcription. In fact, epigenetic mechanisms are likely responsible for mediating the gene-specific, cell-specific, and tissue-specific gene transcription of nuclear receptors, and therefore, may be a potential mechanism exploited for therapeutically targeting nuclear receptors for the prevention and treatment

of disease. However, until the recent development of genome-wide binding technology, understanding the role of epigenetics in regulating nuclear receptor function on a genome-wide scale has been lacking. This comprehensive understanding of genome-wide nuclear receptor function is critical because of the major impact it has on developing selective nuclear receptor modulators.

FXR is a type II nuclear receptor serving as a master regulator of bile acid homeostasis. FXR is highly expressed in the liver and intestine and, as mentioned, has been shown to protect against several liver and intestinal diseases. In order to further determine the potential of therapeutically targeting FXR, epigenetic mechanisms involved in regulating the genome-wide tissue-specific functions of FXR and in regulating the transcription of FXR itself, needs to be further elucidated, and was the focus of this dissertation. The results from these studies introduced novel epigenetic mechanisms involved in regulating tissue-specific functions of FXR and presents DNA methylation of the FXR gene promoter as the epigenetic mechanism responsible for silencing FXR expression in human colon cancer.

7.2 Overall Conclusions:

7.2.1 Genome-wide binding of FXR in liver and intestine.

As mentioned, a comprehensive genome-wide binding profile of FXR is necessary to begin to understand the genome-wide effects of epigenetics on FXR induced transcription. We began this process by performing a genome-wide binding assay of FXR in mouse livers and intestines after ligand treatment. This study revealed novel information on how FXR regulates gene transcription in a genome-wide, tissue-

specific manner. First, this study confirmed a high degree of tissue-specific binding of FXR, with the liver and intestine sharing only a small percentage common FXR binding sites. In addition, this analysis showed novel FXR binding sites located large distances upstream (intergenic), downstream, or within genes (intragenic). Even though a large portion of FXR binding sites are located more than 10 kb away from a RefSeq gene, indicating long-distance chromatin looping as a possible mechanism for FXR-mediated regulation of gene transcription, the majority of FXR binding sites are concentrated within coding regions of the mouse genome. Of these sites, FXR is highly associated with the proximal promoter and first intron of genes.

Further analysis revealed novel FXR binding patterns within known and unknown FXR target genes. For example, FXR was shown to bind to the 5' promoter and 3' enhancer region of the Shp gene (*Nr0b2*). This novel binding pattern was further determined to form a head-to-tail chromatin loop around the Shp gene which is mediated by the activation of FXR (Li et al., 2010). Interestingly, this novel binding pattern was found within other known FXR target genes, such as *Ostb* and *Bsep*, suggesting FXR mediates a head-to-tail chromatin loop around these genes as well. Furthermore, novel FXR binding sites were found within previously uncharacterized target genes, including Pxr, Rarα, and Ntcp, suggesting alternative physiological roles of FXR in drug, steroid/hormone, and even bile acid metabolism than have not been previously characterized.

Pathway analysis of FXR binding in liver and intestine revealed that FXR plays different physiological roles in liver and intestine. In addition, motif analysis identified a potential tissue-specific motif recognized by FXR. This analysis indicates the most

represented FXR binding motif in mouse liver is an IR-1, which has been identified in many FXR target genes. In mouse intestine, an IR-1 and an ER-2 motif were represented as possible FXR binding sequences, suggesting a possible tissue-specific motif recognized by FXR. In addition, there was a half nuclear receptor binding site adjacent to the FXR binding site in both liver and intestine. Several orphan nuclear receptors have been shown to bind to this particular half site suggesting a potential interaction between FXR and other orphan nuclear receptors/TFs to regulate tissue-specific functions of FXR (Catalano et al., 2010; Chong et al., 2010; Ito et al., 2000; Wilson et al., 1992). Furthermore, the interaction between hormone nuclear receptors and other TFs, such as pioneer factors, have been previously reported. For example, ERα has been shown to interact with FOXA1 in order to initiate transcription of ER target genes (Lupien et al., 2008).

In conclusion, this study analyzed the genome-wide binding of FXR in mouse liver and intestine after ligand treatment. The broad distribution of FXR binding, novel FXR binding patterns to known and unknown target genes, and the presence of a nuclear receptor half site associated with FXR binding, all suggest epigenetic mediators responsible for regulating tissue-specific functions.

7.2.2 FXR and HNF4α interact to cooperatively regulate gene transcription in the liver.

As has been thoroughly discussed, results from genome-wide binding analysis of FXR in liver and intestine suggest the involvement of orphan nuclear receptors in regulating FXR function. HNF4α is an orphan nuclear receptor essential for regulating

many liver-specific functions including development, regeneration, production of clotting and serum factors, lipoprotein synthesis, and drug metabolism (Gonzalez, 2008). In addition, HNF4α has been shown to cooperatively regulate the liver-specific functions of the type II nuclear receptors, PXR and CAR (Tirona et al., 2003). However, whether or not HNF4α regulates the liver-specific functions of FXR has not been determined. Therefore, the effect of HNF4α on FXR binding and transcriptional activity in mouse liver was assessed.

To begin, a genome-wide binding analysis of FXR and HNF4α in mouse livers was done. This analysis showed a high percentage of FXR binding sites in liver co-localized with those of HNF4α. In addition, binding frequency of HNF4α to shared target sites was greatest when bound upstream and in close proximity to FXR, suggesting a possible interaction between FXR and HNF4α, and implicating they function to co-regulate gene transcription. Furthermore, preliminary ChIP-qPCR analysis revealed that binding of HNF4α to shared FXR target genes was dependent on the presence and activity of FXR, but FXR binding was not dependent on the presence of HNF4α. Likewise, co-IP assays indicate a protein-protein interaction between FXR and HNF4α, which is increased upon FXR activation. Finally, HNF4α was shown to transcriptionally regulate the FXR target gene SHP by moderately increasing FXR transcriptional activity of the SHP gene.

These results revealed only a weak FXR-HNF4α interaction, and only a moderate cooperative effect of HNF4α on transcriptional activity of FXR. However, the effects of HNF4α on FXR function could be more profound than was represented in these assays. For example, HNF4α binding co-localized with FXR to both the 5’

promoter and 3' enhancer region of the SHP gene. As mentioned, binding of FXR to the 5' and 3' region of the SHP gene mediates a head-to-tail chromatin loop around the SHP gene (Li et al., 2010). This may be an essential epigenetic mechanism critical for FXR induced transcription of the SHP gene. This same binding pattern of HNF4α to the SHP gene implicates that HNF4α may be critical in mediating this head-to-tail chromatin loop induced by FXR activation, which is a physiological mechanism not reflected in the assays described above.

Nevertheless, this study ultimately confirmed a cooperative effect of FXR and HNF4α in mouse liver; thereby suggesting HNF4α could be an important nuclear receptor critical for regulating the liver-specific functions of FXR on a genome-wide scale. These results help progress the field focused on designing FXR modulators for the treatment of liver diseases.

7.2.3 DNA methylation as a mechanism of FXR down-regulation in human colon cancer.

Colon cancer is a significant public health issue in the United States. Even though a portion of colon cancer cases are due to heritable DNA mutations, the majorities of cases are sporadically derived and arise due to environmental causes. The western high-fat, low-fiber diets have been associated with increased colon tumorigenesis (Armstrong and Doll, 1975). This has been partially attributed to an increased exposure of colonic epithelium to bile acids. Increased fat consumption causes an increased intestinal load of bile acids, and low-fiber intake increases

gastrointestinal transit time, thereby, increasing both level and time of bile acid exposure (Correa, 1981; Willett et al., 1990).

Due to their hydrophobicity and detergent-like properties, secondary bile acids DCA and LCA, have been shown to increase intestinal epithelium cytotoxicity and promote cell proliferation, and therefore, have been linked to increased colon carcinogenesis (Lechner et al., 2002). However, under normal physiological conditions, cellular mechanisms in intestine and liver exist to increase detoxification of bile acids in order to prevent their accumulation to cytotoxic levels. This process, which has been extensively discussed, is regulated by FXR, as well as other nuclear receptors. FXR regulates nearly every aspect of the enterohepatic circulation of bile acids including the synthesis, efflux, intracellular trafficking, and enzymatic metabolism of bile acids (Inagaki et al., 2005; Okuwaki et al., 2007; Sinal et al., 2000; Tu et al., 2000; Zollner et al., 2006), and therefore, has been suggested to protect against the development of colon cancer. In fact, mice deficient in FXR have increased colon tumorigenesis (Maran et al., 2009; Modica et al., 2008). However, FXR clearly has anti-tumorigenic effects aside from its role in regulating bile acid levels (Inagaki et al., 2006; Modica et al., 2008). Ultimately, because of its anti-tumorigenic effects, FXR has become a promising therapeutic option for the treatment and/or prevention of colon cancer.

This study determined FXR expression in human colon cancers and in intestine of APC^{min} mice, a common mouse model for intestinal cancer, was decreased. Likewise, FXR expression in human colon cancer cell lines was inversely associated with malignancy of cancer cell. Sequencing of the FXR gene revealed no mutations that could account for differences in FXR expression or function in human colon cancer.

However, treating colon cancer cells with a DNMT inhibitor significantly increased FXR expression, suggesting DNA methylation as an epigenetic mechanism responsible for silencing FXR expression in colon cancer. Bisulfite sequencing and MeDIP analysis confirmed a CpG island located in the FXR promoter and the degree of FXR promoter methylation inversely correlated to expression of FXR in human colon cancer cells. Finally, siRNA knockdown of DNMT 1 and 3B resulted in an increase of FXR expression in the colon cancer cell line SW620 suggesting these enzymes are responsible for methylating FXR promoter, leading to decreased expression.

Interestingly, little is known about the role of other TFs in regulating the expression of FXR in the intestine. However, results from this study also suggested the involvement of a factor(s) that may be responsible for regulating FXR expression in the intestine, which is silenced by DNA methylation in human colon cancer. Nevertheless, more research needs to be done to further elucidate this mechanism.

In conclusion, this study determined that FXR is silenced in human colon cancer and that the epigenetic mechanism involved in FXR silencing is partially due to DNA methylation of the FXR promoter. Therefore, targeting FXR for treatment and/or prevention of colon cancer may be hindered due to selective down-regulation of FXR. However, reversing the silencing of FXR in human colon cancer should be further investigated as a potential therapeutic option for slowing the progression of colon cancer.

7.3 Concluding Remarks:

Combined, this dissertation revealed a high degree of genome-wide tissue-specific binding of FXR proposed to be regulated by epigenetic factors, that HNF4α cooperatively regulates the liver-specific functions of FXR, and that DNA methylation of the FXR promoter is the epigenetic mechanism partially responsible for silencing FXR in human colon cancer. Genome-wide binding analysis predicted orphan nuclear receptors are responsible for regulating tissue-specific transcription of FXR target genes. HNF4α is an orphan nuclear receptor shown to moderately enhance FXR function in liver, and may be an important factor critical for regulating liver-specific function of FXR. Finally, FXR expression is decreased in human colon cancer and an epigenetic mechanism, namely DNA methylation, is responsible for the silencing of this gene. These results reveal novel information on the role of epigenetics in regulating FXR induced transcription and expression of FXR in human colon cancer, which greatly advances the basic knowledge needed to enhance development of selective FXR modulators for the prevention and treatment of human diseases.

Chapter 8: Future Directions

The results from my graduate research were very exciting because they have begun to explain epigenetic mechanisms involved in FXR regulated transcription of target genes and in silencing of FXR in colon cancer. However, much work is needed to fully characterize the intricately regulated mechanisms involved in FXR function. For this reason, there are several directions in which this research could be continued.

First, genome-wide binding analysis of FXR in this dissertation focused mainly on FXR expressed in mouse liver and intestine. Likewise, cooperative interactions between FXR and HNF4α were also investigated in mouse liver. Throughout this dissertation, I have extensively discussed how FXR has become a potential therapeutic target for treating multiple *human* diseases. Therefore, information obtained from FXR function in mice needs to be translated for understanding FXR function in humans. Although mice are traditionally used as models for human diseases, species differences may exist for FXR function. Therefore, a genome-wide binding analysis of FXR in human will be of great help in understanding the similarities and differences between mouse and human FXR function. One of the limitations for this is finding a representative human model to use for genome-wide binding analysis. Many genome-wide binding studies have looked at TFs within human cancer cell lines, which is ideal for obtaining a genetically homogenous cell population. One might argue, however, that cancer cells displayed deranged cellular function, and may not represent normal physiological functions of FXR. Conversely, human tissues obtained from hospital tissue banks may be a way to investigate normal physiological functions of FXR. However, genetic variability and

environmental exposure from one patient to the next would likely be an obstacle. Nevertheless, there is a clear need for the information gained from genome-wide binding analysis in mice to be translated into humans. Therefore, this is a likely future direction for this project.

Furthermore, even though FXR is highly expressed in liver and intestine, FXR is also expressed to a lesser extent in other tissues such as the adrenal gland, kidney, heart, and adipocytes (Zhang et al., 2003). However, little is known about the role FXR plays in these secondary tissues, although it is suggested that FXR functions to significantly regulate systemic metabolic parameters. As was clearly demonstrated in this dissertation, genome-wide binding of FXR was highly tissue-specific. It would be a critically important endeavor to investigate FXR's role on a genome-wide scale within different systemic tissues. The information gained from this would assist in piecing together FXR's systemic function within an organism as well as give insight into systemic off-target effects that would be associated with pharmacologically targeting FXR. Furthermore, it may be determined that targeting FXR for the treatment of disease could be extended to other tissues such as the adrenals and kidney, although FXR's role in these tissues is poorly understood.

In addition to investigating species and tissue differences in genome-wide binding of FXR, more work needs to be done to elucidate the role of FXR in regulating gene expression of 3-dimensional chromatin. ChIP-seq of FXR unveiled some suggestive ideas of how FXR regulates gene expression in a 3-dimensional manner. However, how TF regulate 3-dimensional chromatin interactions is crucial for understanding regulation of gene transcription. ChIP-seq analysis identified a high

degree of distant FXR binding sites, but determining the function of binding to these regions needs to be investigated. High-through-put technology has recently been developed in order to capture chromatin in a 3-dimensional structure and detect long-range chromatin interactions. This experimentation includes cross-linking nuclear chromatin, fragmenting chromatin by sonication, immunoprecipitating chromatin with antibody against TF of interest, and tethering immunoprecipitated DNA elements in close special distances through proximal ligation with paired-end linker tags. This is followed by restriction enzyme digestion and reverse-cross linking and the newly tethered DNA fragments are prepared for genome-wide sequencing analysis (Li et al., 2010). This new technology is referred to as chromatin interaction analysis with paired-end tag sequencing (ChIA-PET). This is certainly a potential direction to pursue in order to fully characterize the effects of FXR function in 3-dimensional genome-wide scale.

There is also a need to confirm and further elucidate the role of HNF4α in regulating FXR function in human hepatocytes. This dissertation has already described the effects of HNF4α on FXR function within mouse livers and in an *in vitro* system, and HNF4α was determined to have only a modest effect on FXR activity. Ideally, this association needs to be confirmed *in vivo* within human hepatocytes. However, an even more intriguing and pressing issue is to investigate the role of other orphan nuclear receptors in regulating FXR function. The reasoning behind this, in terms of targeting FXR function therapeutically, is that HNF4α is an extremely abundant protein within hepatocytes, serves many important hepatocyte-specific functions, and only appears to have a modest effect on FXR function. Therefore, targeting HNF4α to alter FXR function, or targeting HNF4α therapeutically at all, may be associated with many undesirable off-

target effects, possibly more adverse side-effects than targeting FXR alone, and therefore, serves as a poor therapeutic option. However, there are likely other orphan nuclear receptors, whose functions are poorly understood, which serve to regulate FXR function. For example, as discussed above, genome-wide binding analysis revealed a nuclear receptor half site associated with FXRRE in mouse liver and intestine (Thomas et al., 2010). LRH-1 is an orphan nuclear receptor shown to bind to this half site and synergistically enhance transcriptional activity of FXR (Chong et al., 2010). However, other nuclear receptors are capable of binding this nuclear receptor half site and could function to regulate FXR activity (Harding et al., 1997; Harding and Lazar, 1993; Wilson et al., 1993; Wilson et al., 1992). Therefore, another future direction would be to elucidate existing orphan nuclear receptors within the liver and intestine that regulate FXR function.

Finally, I have described in this dissertation how FXR is down-regulated in human colon cancer, in part, by DNA methylation. Furthermore, I have extensively discussed how FXR deficiency in mice is associated with increased intestinal tumorigenesis (Maran et al., 2009; Modica et al., 2008), suggesting FXR as tumor suppressor. Studies have been done demonstrating activation of FXR attenuates intestinal symptoms in mouse models of colitis and irritable bowel disorder (Vavassori et al., 2009). It has been speculated that activation of FXR would also decrease intestinal tumorigenesis, though this has not been sufficiently studied. Therefore, a future direction for this project would be to test whether FXR activation by a synthetic agonist would decrease tumorigenesis in mouse models of intestinal cancer. I would further like to investigate the mechanism of how FXR decreases intestinal tumorigenesis. As

discussed above, FXR likely suppresses tumorigenesis via two methods, through preventing bile acid accumulation to toxic concentrations and by directly regulating cell proliferation and/or apoptosis, independent of its role in bile acid homeostasis. My goal would be to fully elucidate FXR's anti-tumorigenic role. Furthermore, an ideal outcome for this project would be to propose FXR as a therapeutic target for chemoprevention and/or treatment of human colon cancer. Therefore, it is my goal to conduct a clinical study using an FXR agonist, whether synthetic or natural, in patients who are at high risk for colon cancer development, and determine the degree of FXR-induced cancer prevention or suppression.

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