GENE SELECTIVE REGULATION BY HEPATIC FARNESOID X RECEPTOR (FXR) IN HEALTH AND DISEASE

BY

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DISSERTATION

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ABSTRACT

Metabolic syndrome is a clustered condition including obesity, insulin resistance, hypertension, dyslipidemia, and liver steatosis. The incidence of metabolic syndrome and the accompanying risk of developing cardiovascular disease and type II diabetes is dramatically increasing. Disrupted homeostasis of metabolites contributes to metabolic disease. Farnesoid X Receptor (FXR) which is known as a primary bile acid nuclear receptor plays important roles for maintaining levels of metabolites like triglycerides, fatty acids and glucose by regulating its targets genes in liver. Over the past decades, compelling studies establishing the function of FXR were performed using a synthetic FXR ligand, GW4064 and FXR null mice. These studies revealed that FXR inhibits triglyceride biosynthesis and glucose production to regulate glucose and lipid homeostasis. However, it is not known how FXR regulates its targets in metabolic diseases, like fatty livers associated with elevated triglyceride and glucose levels. As a ligand-dependent transcriptional regulator, FXR regulates numerous target genes by binding to FXR-responsive DNA elements (FXRE) with a heterodimer partner, RXRα, in response to bile acids or other physiological stimuli. Interestingly, our group reported that FXR is aberrantly acetylated in fatty liver induced by a high fat diet, and acetylation of FXR impaired its ability to form a heterodimer with RXRα or to bind to DNA. Taken together, these observations led us to ask how FXR maintains metabolic homeostasis by regulating its target genes in normal and disease state. Despite the known FXR functions in liver metabolism, whether FXR mediated transcriptional responses are altered in metabolic disease state is not known. To begin to address this question, I analyzed FXR regulation of miRNAs by gene array analysis and binding of FXR genome-wide by ChIP-seq studies.
FXR regulates many target genes in the liver in different metabolic pathways. It is not clear how this is accomplished, but one possibility is that FXR regulates expression of miRNAs which are ideal candidates to regulate expression of multiple groups of genes. miRNA-mediated regulation by hepatic FXR were examined by miRNA-microarrays using livers of FXR null mice. miRNAs are small non-coding regulatory RNAs which act by binding to 3’-untranslated regions (UTRs) of target mRNAs to regulate expression of the gene products. In this study, the most significantly downregulated miRNA in FXR null mice was miR-34a, which was also shown to be abnormally expressed miRNA in human liver steatosis. FXR activation by bile acid treatment resulted in decreased miR-34a levels through induction of small heterodimer partner (SHP). miR-34a inhibits expression of SIRT1 which is a key metabolic regulator as well as the best known longevity molecule at the post-transcriptional level. Theses events link SIRT1 regulation by FXR to miR-34a inhibition in healthy liver. Interestingly, this molecular link was impaired in the metabolic diseased liver which has markedly elevated miR-34a levels and reduced SIRT1 levels. This study provides evidence for the regulation of the levels of SIRT1, a metabolic switch, by FXR through repression of miRNA expression in healthy liver. Furthermore, this finding supports the idea that activation of FXR by ligand treatment in diseased mice positively regulates glucose and lipid homeostasis by increasing hepatic SIRT1 levels via miR-34a suppression.

In liver disease states such as fatty liver, FXR is hyperacetylated and regulation of FXR target genes is altered. It is possible that modification of FXR may affect its binding to its target genes. To determine whether hepatic FXR differentially interacts with target binding sites in the metabolic disease state compared to healthy states, genomic FXR binding sites in normal and fatty livers were examined by ChIP-sequencing. The total number of FXR binding sites detected
was 5,272 in fatty livers induced by high fat diet (HFD), compared to 15,263 in normal diet (ND) livers. The differences in number of binding sites may be partially explained by reduced FXR levels, reduced DNA binding of FXR by acetylation in the metabolic disease liver or by experimental variations including false positive signals. Most of the FXR binding sites were located in intergenic- or intronic region with an IR-1 motif (AGGTCA) in both diet groups. Interestingly, 7,440 or 2,344 unique FXR binding sites were identified in ND or HFD, respectively. Gene ontology (GO) annotated these unique FXR binding sites to groups of genes including Ca²⁺/K⁺ channels, S/T kinases, and lipid oxidation genes in the ND group and iron-sulfur binding proteins, Toll-like receptors, and oncogenic proteins in the HFD group. Randomly selected genes occupied by FXR uniquely in either ND or HFD were analyzed by standard ChIP and 90% of FXR binding sites detected in the ChIP-seq studies were validated in for ChIP analysis. In addition, altered gene expression was associated with FXR binding suggesting that FXR binding is functionally significant and that FXR regulates different sets of genes in ND or HFD mice.

The miRNA studies advance our understanding of how FXR regulates its target genes using miRNAs to maintain metabolic homeostasis for healthy liver and how the regulation is abnormal in metabolic disease states. In addition, genomic FXR binding analysis broadened our insight into the biological functions of FXR suggesting that FXR is highly involved not only in the liver metabolism but also in the inflammation, apoptosis, and liver cancer. Importantly, these findings may provide useful information for the development of therapeutic reagent treating liver diseases, metabolic syndrome.
Acknowledgements

I deeply thank my God, my lord for all the things I have and will have.
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Chapter One

Gene regulation by hepatic FXR in health and disease:
Background and Significance

Introduction
I. FXR as a Nuclear Bile Acid Receptor

Nuclear receptors (NRs) are transcriptional factors that regulate the activity of genetic networks involved in several diverse physiological functions including reproduction, development and metabolism in response to signaling molecules including steroids, retinoids, fatty acids, and bile acids (1). The NRs can be broadly divided into three sub-groups based on their physiologic ligands and potential functions (2). The first class of NRs is the classic endocrine receptors characterized by their very high affinity to ligands. This class of receptors is essential for homeostatic regulation of the endocrine system, and includes steroid hormone receptors, such as estrogen receptor (ER), and vitamin A and D receptors. The second and third classes of NRs are orphan NRs which are identified based on their sequence homology to the endocrine NRs, but originally lacked a cognate ligand (3). The concept of reverse endocrinology, in which a receptor is used to discover its natural ligand, was used to characterize the functions of the second class of NRs (4). Many endogenous and exogenous compounds have been identified as ligands for some orphan NRs and these are called deorphanized or adopted. The adopted NRs belong to the second class of NRs showing low affinity to their ligands (5-9). These NRs are also referred to as metabolic receptors, since they are essential in regulating lipid/glucose and xenoliths uptake, synthesis, storage and clearance, although the nature of some adopted receptors has not been characterized (1, 9-11). The third class of NRs contains all the remaining true orphan NRs, whose ligands have not yet been identified.
Farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily and is designated NR1H4 (1). It is highly conserved from teleost fish to humans suggesting a critical role of FXR in numerous species (1-5). The single human or mouse gene encodes four isoforms, FXRα1, FXRα2, FXRα3, and FXRα4. The latter two isoforms are also referred to as FXRβ1 and FXRβ2 (12). Compared to FXRα1 and FXRα2, FXRα3 and FXRα4 mRNAs are smaller as a result of the use of two different promoters but encode larger proteins that contain an extended N-terminus (Fig. 1-1). In addition, FXRα1 and FXRα3 have an insert of four amino acids (MYTG) immediately adjacent to the DNA-binding domain (DBD) in a region referred to as the ‘hinge region’ as a result of alternative splicing between exons 5 and 6. The main FXR target genes are regulated in an isoform-independent manner, however, it is known that a few genes including those encoding intestinal bile acid binding protein (IBABP), syndecan-1, αA-crystallin and fibroblast growth factor 19 (FGF19) are more responsive to the FXRα2 and FXRα4 isoforms lacking the MYTG motif than to FXRα1 and FXRα3 (1, 12). The physiological importance of gene activation by specific FXR isoforms remains to be established.

FXR is highly expressed in the liver, intestine, kidney and adrenal gland, with much lower levels in adipose tissue (5). In general, NRs bind as monomers or dimers to specific DNA sequences termed hormone response elements and regulate gene expression (4,11). Like many other non-steroid hormone NRs, FXR binds to specific DNA elements called FXR response elements (FXRE) as a heterodimeric complex with 9-cis-retinoid X receptor (RXR) (13). The FXRE contains two copies of a consensus sequence (AGGTCA) arranged as inverted repeats separated by one nucleotide (IR1), everted repeats separated by 8 nucleotides (ER9) or direct repeats separated by four nucleotides (DR4) (12,13). FXR also has been reported to bind DNA as monomer to represses its target genes, although this appears to be a rare event (14).
Fig. 1-1. Genomic organization and four isoforms of FXR.  
a. Structure of the FXR gene. The 11 exons of the mouse and human FXR genes are indicated along with the two functional promoters that initiate transcription at exons 1 or 3. Alternative splicing of the initial RNAs produces four mRNAs. The alternative splicing of the 12 bp at the 3’ end of exon 5, which encode the MYTG motif, is in dark blue box. Asterisks indicate the translational start sites (ATG). b. The four FXR protein isoforms with the different domains color-coded. AF, activation function: DBD, DNA-binding domain: LBD, ligand-binding domain: MYTG, four amino acids inserted.

Ligands of FXR. Transcriptional activation is often stimulated when agonists, usually small lipophilic molecules, bind to the pocket formed by the ligand binding domain (LBD). Ligand binding alters the conformations of NRs which then bind to their response elements on the target gene promoters (11). This often results in release of corepressors, recruitment of coactivators, and activated gene transcription. In the absence of a ligand, a NR is generally associated with corepressor proteins and is inactive (1, 2). Specific agonists include steroid hormones, thyroid
hormone, 1,25-dihydroxyvitamin D$_3$, fatty acids, oxysterols, retinoic acids, phospholipids and bile acids, while some NRs function independently of a ligand.

Bile acids have been identified as the endogenous ligands that bind to and activate FXR (14-16). The order of potency of endogenous bile acids is chenodeoxycholic acid (CDCA) > lithocholic acid (LCA) = deoxycholic acid (DCA) > cholic acid (CA). Synthetic FXR-specific agonists, such as GW4064, fexaramine, and 6α-ethyl-chenodeoxycholic acid (ECDCA) have been developed (15). Generation of synthetic specific FXR agonists has facilitated investigations the functions of FXR in controlling diverse metabolic pathways.

II. Functions of FXR in liver

Bile acid biosynthesis. As end products of cholesterol catabolism in liver, bile acids play important roles for lipid digestion and adsorption of lipids from the intestinal lumen by serving as signaling molecules (14-16). They also represent the principal means of eliminating cholesterol form the body.

Bile acid synthesis in the liver is tightly regulated by negative feed back mechanisms that decrease the expression of CYP7A1, which is a key enzyme converting cholesterol to bile acids. The mechanism involves bile acids that are absorbed in the small intestine and return to the liver and activate FXR. The activated FXR then binds to the SHP promoter which contains FXR response elements (FXREs) and induces SHP expression. SHP in turn binds another nuclear receptor LRH-1, which is required for CYP7A1 transcriptional activation, thereby inhibiting bile acid synthesis. Mice lacking FXR show increased CYP7A1 expression and elevated bile acid levels (15,16). It is known that bile acids are activators of nuclear receptors not
only for FXR, but also for pregnane X receptor (PXR), vitamin D receptor (VDR), and the constitutive androstan receptor (CAR) (14).

**Lipid and glucose homeostasis.** The nuclear bile acid receptor, FXR plays an crucial role in maintaining lipid and glucose levels by regulating expression of numerous metabolic genes in the liver (17). The activation of FXR by an agonist results in reduced plasma triglyceride, hepatic lipogenesis, cholesterol levels, synthesis of free fatty acids (FFAs), and the secretion of VLDL from liver (18). Increased LDL and induction of genes involved in lipoprotein metabolism and clearance by FXR activation is entirely consistent with previous findings of its role in controlling plasma lipids (17-19).

In addition, FXR exerts an important role in glucose homeostasis. FXR deficiency in mice leads to impaired glucose tolerance and insulin resistance (17,20). Activation of hepatic FXR regulates gluconeogenesis, glycogen synthesis and insulin sensitivity. Treatment of mice with the FXR agonist, GW4064 or cholic acid (CA), or following infection with adenovirus that expresses a constitutively active FXR-VP16 fusion protein, resulted in a significant reduction of plasma glucose levels and improved insulin sensitivity (21). However, the precise role of FXR in the regulation of hepatic glucose metabolism is not still well understood.

Consistent with previous data, disruption of the FXR gene in transgenic mice was associated with metabolic diseases including hypercholesterolemia, hyperglycemia, cholesterol gallstone disease, fatty liver, and type II diabetes (17-20). FXR null mice have a pro-atherogenic lipoprotein profile including increased plasma triglycerides, free fatty acids and LDL suggesting that these mice may have altered susceptibility to atherogenesis (17,18). They also have reduced hepatic expression of scavenger receptor, type I B1 (SR-BI), which is thought to be important in
the delivery of HDL cholesterol to the liver as part of the reverse cholesterol transport pathway.
In addition, reduced hepatic glycogen content and insulin resistance was found in the FXR null
mice compared to wild type mice.

FXR activation has provided beneficial effects in several experimental rodent models (11).
Recent studies showed that activation of FXR repressed hepatic phosphoenoyl pyruvate
carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) mRNA levels (22). This result
suggests that hepatic glycogen synthesis might be altered by FXR. Activation of FXR in the
livers of diabetic mice resulted in increased conversion of glucose to glycogen and increased
hepatic glycogen levels by increasing phosphorylation of GSK3β, a known key regulator of
glycogen synthase (22).

Both GW4064 and chenodeoxycholic acid (CDCA) protect hepatocytes from cholestasis
induced by α-naphthylisothiocyanate and estrogen (23). In addition, activation of FXR in
diabetic obese mice by long term treatment with GW4064 improved metabolic outcomes by
reducing serum glucose and lipid levels (21). These studies suggest that changes in FXR
expression and activity may affect numerous genes that are associated with metabolic diseases.
Together, the current data suggest that FXR agonists may be useful in the treatment of type 2
diabetes, hypertriglyceridemia, certain cholestasis conditions, and cholesterol gallstone disease.
Although FXR has been shown to be critical for hepatic metabolism, and activation of FXR
improves metabolic outcomes in diet-induced obese mice (17,18, 21, 22), it is unclear how FXR
modulates or regulates liver metabolic target genes.

III. Cofactors of FXR function
**Cofactors of FXR.** According to the general model for NR function, in the absence of an agonistic ligand or in the presence of an antagonistic ligand, the C-terminal activation domains of NRs associate with transcriptional corepressors (4,10,15). Upon binding to an agonistic ligand, the C-terminal domain undergoes a conformational shift, leading to dissociation of corepressors and association with transcriptional coactivators. Many of these coactivators act by acetylating histones or non-histone proteins in their vicinity, consequently leading to an enhancement of gene transcription (23). Coregulators working with DNA-bound transcription factors (TFs) may enhance or repress transcription and can be classified into distinct groups, such as bridging factors, protein-modifying enzymes, chromatin remodeling complexes or mediator complexes, based on their biochemical and functional properties (24).

The FXR coactivator, DRIP205A, a subunit of the vitamin D receptor interacting protein (DRIP)/thyroid hormone receptor associated protein (TRAP) coactivator complex, was shown to interact with FXR in a ligand-dependent manner (24). DRIP205A increases transactivation of FXR response element-driven reporter constructs in the presence of exogenously expressed FXR and its heterodimerization partner RXR (25). Another coactivator, peroxisome proliferator-activated receptor-γ coactivator-1 α (PGC1α), was shown to interact with the DNA binding domain of FXR in a ligand-dependent manner to activate FXR function through coactivation of the nuclear receptors PPARγ and HNF-4α (26). FXR has been shown to interact with Baf60c, a subunit of the ATP-dependent SWI/SNF chromatin remodeling complex in a ligand dependent manner (27). In addition, it was reported that p300 directly interacted with and acetylated FXR in vitro (28). FXR interacts with p300 and recruits it to the SHP promoter resulting in acetylation of histones at the promoter in response to FXR agonists in mouse liver and human hepatoma cells (28). Recently, our group has reported that FXR is a target of SIRT1,
a deacetylase that mediates nutritional and hormonal modulation of hepatic metabolism (29). Lysine 217 of FXR is the major acetylation site targeted and is regulated dynamically by p300 and SIRT1, but is constitutively elevated in metabolic disease states (29). Cofactors allow FXR to act as a sensor of cellular metabolism and consequently orchestrate rapid responses in specific gene expression to the metabolic or toxicological information in the cellular environment.

**SRT1, a FXR coactivator.** SIRT1 is a NAD\(^+\)-dependent histone and protein deacetylase (30). It was identified as a mammalian homolog of yeast Sir2 protein which is known as a longevity molecule in worms (30, 31). It is well known that SIRT1 alters the activities of transcriptional factors which regulate metabolic genes (30,31). SIRT1 also mediates the beneficial metabolic effects of caloric restriction (CR) (32). It deacetylates and regulates the transcriptional abilities of metabolic regulators, such as PGC-1\(\alpha\), p53, Foxo 1, NF-\(\kappa\)B, LXR, and FXR that are involved in lipid and glucose metabolism, inflammation, mitochondrial biogenesis, and energy balance (30-35).

Beneficial metabolic functions of SIRT1 have been demonstrated in several studies using small molecule activators. The natural compound resveratrol and the synthetic compound SRT1720 are activators of SIRT1 and have been shown to ameliorate insulin resistance, increase mitochondrial content, improve metabolic profiles, and increase survival in mice fed a high-fat diet (35-40). All these recent studies demonstrate that SIRT1 is a key regulator of cellular metabolism and mediates beneficial metabolic effects.

**IV. MicroRNAs in metabolism**
A post-transcriptional regulator, microRNA. MicroRNAs (miRNAs) are small (approximately 22 nt) non-coding RNAs that control gene expression (41). MiRNAs are transcribed from DNA by RNA polymerase II as hairpin precursors which are further processed by Drosha and Dicer to mature forms. MiRNAs bind to the 3’-untranslated region (UTR) of target mRNAs and inhibit their expression by causing mRNA cleavage or inhibition of translation. Approximately 30% of all human genes are thought to be regulated by miRNAs and indeed, miRNAs are known to control gene expression in diverse biological processes including development, differentiation, cell proliferation, and apoptosis (42-54).

MiRNAs in metabolic regulation. Crucial roles of miRNAs in the regulation of cellular metabolism have been demonstrated recently as summarized in Table 1-1. A number of the miRNAs are involved in lipid and glucose metabolism in major metabolic tissues such as liver, pancreas, adipose, and muscle. The most abundant miRNA in liver, miR-122 plays important roles in a wide variety of liver functions ranging from cholesterol metabolism, liver cancer, stress responses, and viral infection to circadian regulation of hepatic genes (42,43). MiR-33 has been shown to contribute to the regulation of cholesterol homeostasis by targeting the cholesterol transporter genes, ABCA1 and ABCG1 (45,46). MiR-34a was also shown to suppress insulin secretion in pancreatic β-cells in response to glucose (47). The roles of miR-375 in pancreatic islet functions, especially in insulin gene transcription, insulin secretion, and islet cell growth, have also been well established (48,49). For lipid metabolism, miR-27 and miR-378 were reported to control adipocyte differentiation and lipid synthesis, respectively (50,51). In heart, miR-223 was shown to regulate glucose uptake in cardiomyocytes, and miR-696 was shown to regulate mitochondria biogenesis and fatty acid oxidation in gastrocnemius muscle (52,53). In line with their critical functions, miRNAs are often underexpressed or overexpressed in disease
Recent studies have shown that restoring miRNAs or downregulating miRNAs using antisense miRNA inhibitors, called Antagomirs, may result in improved transcriptional and biological outcomes, demonstrating that miRNAs are promising therapeutic targets (41).

Table 1-1. MicroRNAs regulating cellular metabolism in major metabolic tissues.

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Direct [potential] targets</th>
<th>Functional roles in Metabolism</th>
<th>Tissues (Cell lines)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-33</td>
<td>ABCA1, NPC1</td>
<td>Cholesterol homeostasis</td>
<td>Livers (HepG2)</td>
<td>42,43,44,45,46</td>
</tr>
<tr>
<td>miR-34a</td>
<td>SIRT1</td>
<td>Hepatic Lipid metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Islet beta-cell exocytosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell apoptosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-370</td>
<td>Cpt1a</td>
<td>Fatty acid and Triglyceride biosynthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-122</td>
<td>CAT-1, ADAM17</td>
<td>Lipid metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-34a</td>
<td>VAMP2</td>
<td>B-cell exocytosis</td>
<td>Pancreas Islets (MIN6, INS-1)</td>
<td>47,48,49</td>
</tr>
<tr>
<td>miR-124a2</td>
<td>Foxa2</td>
<td>Intracellular signaling in pancreas β-cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-146</td>
<td>VAMP2</td>
<td>B-cell exocytosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-375</td>
<td>MTPN</td>
<td>Regulates catecholamine release, Inhibits insulin secretion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-27a</td>
<td>[PPARγ, C/EBPα]</td>
<td>Inhibits adipocyte formation, Down-regulated during adipogenic differentiation</td>
<td>Adipocyte (3T3-L1, ST2)</td>
<td>50,51</td>
</tr>
<tr>
<td>miR-378/378*</td>
<td>[Ribosomal proteins]</td>
<td>Upregulates adipocyte differentiation and lipid synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-223</td>
<td>Glut4</td>
<td>Glucose uptake and insulin resistance</td>
<td>Muscles (Cardiomyocyte, Gastrocnemius, C2C12)</td>
<td>52,53</td>
</tr>
<tr>
<td>miR-696</td>
<td>[PGC1a]</td>
<td>Muscle metabolism, mitochondria biogenesis and fatty acid oxidation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Adapted from Lee and Kemper, 2010.) (55)

V. Genomics in Nuclear receptors
Many cellular signaling pathways ultimately control specific patterns of gene expression in the nucleus through a variety of signal-regulated transcription factors, including nuclear receptors (23,24,56). A longstanding goal of genomics is to learn how genomes encode the diverse patterns of gene expression that define each cell type and state (24). Genomic assays have allowed the identification of gene sets whose expression is regulated by signaling mediated by nuclear receptors in a particular pathway (24). The advent of genomic technologies for examining signal-regulated transcriptional responses and transcription factor binding on a genomic scale has dramatically increased our understanding of the cellular programs that control cellular signaling and gene regulation (24,53). Currently, hybridization-based and sequencing-based approaches are the two preferred methods to analyze gene expression in a global and high-throughput manner (57).

**Microarrays for analyzing gene expression.** Tiled microarrays are now commonly used for the global analysis of gene expression, as evidenced by the vast number of data sets that have been deposited in public repositories. DNA binding specificity and potential target genes for FXR have been identified using microarray assays (58) which have provided hints of gene regulation mediated by FXR. However, these assays have disadvantages like a narrow dynamic range of detection, a high level of background noise, and in most cases, a limited coverage of the genome, especially for organisms with large genomes (23,24,57).

**Sequence-based approaches for analyzing gene expression.** Although microarrays have been used for most of the global gene expression studies to date, limitations in the technology as mentioned above have stimulated development of alternative methods. One alternative to
hybridization microarrays is massive sequencing like RNA-seq and ChIP-seq (57,59), which are characterized by large genome coverage, quantitative measurement of transcripts levels, higher resolution, and a larger dynamic range. Chromatin Immunoprecipitation (ChIP) is a standard technique for mapping transcriptional factor (TF) binding sites in cells and tissues (24,59). In ChIP assays, chromatin is cross linked chemically, then further precipitated by antibodies of proteins of interest. DNA from chromatin bound by antibody is quantified by semi-quantitative PCR or quantitative real time PCR for gene-specific analysis. By combining the standard ChIP assay with high-throughput genomic technologies, identifying the location of TF binding sites on a genomic scale is now possible. Several platforms are currently available for genomic ChIP, based on either hybridization (ChIP-chip) or deep sequencing (ChIP-seq) (24). Using these techniques, the genomic binding sites of numerous hormone or signal-regulated TFs have been mapped. Genome-wide measurements of protein-DNA interaction by chromatin immunoprecipitation (ChIP) and quantitative measurements of transcriptomes are increasingly used to link regulatory inputs with transcription outputs.

Recently, genome-wide FXR binding sites in liver and intestine with GW4064 for FXR activation have been mapped using ChIP-seq (60). About 1,700 binding sites for FXR in the liver were found upon GW4064 activation, and a motif search suggested that all contain an identifiable IR-1 site (60). Most of the sites are located in intergenic and intronic regions but there is a significant enrichment of FXR-binding sites within 2 kb of transcription start sites (TSS) for known genes. Motif analysis has revealed a half nuclear receptor binding site, normally bound by a few orphan nuclear receptors, adjacent to the FXR response elements, indicating possible involvement of some orphan nuclear receptors like LRH-1 in modulating FXR function (61). It remains to be explored whether FXR differentially binds to its targets in
pathophysiological states, like diet induced obesity. In Chapter III, genome-wide chromatin localization of FXR was examined using ChIP-seq analysis in the livers of normal and diet-induced obese mice.
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Chapter Two

FXR/SHP pathway positively regulates hepatic SIRT1 levels
via miR-34a inhibition

Abstract

SIRT1 is a NAD-dependent deacetylase that is critically involved in diverse cellular processes including metabolic disease, cancer, and possibly aging. Despite extensive studies on SIRT1 function, how SIRT1 levels are regulated remains relatively unknown. Here we report that the nuclear bile acid receptor FXR inhibits microRNA-34a (miR-34a) in the liver, which results in a positive regulation of SIRT1 levels. Activation of FXR by the synthetic agonist GW4064 decreases hepatic miR-34a levels in normal mice and consistently, hepatic miR-34a levels are elevated in FXR null mice. FXR induces expression of SHP, an orphan nuclear receptor and transcriptional corepressor, which in turn results in repression of p53, a key activator of the miR-34a gene, by inhibiting p53 occupancy at the promoter. MiR-34a decreased SIRT1 levels by binding to the 3’ untranslated region (UTR) of SIRT1 mRNA and adenoviral-mediated overexpression of miR-34a substantially decreased SIRT1 protein levels in mouse liver. Remarkably, miR-34a levels were elevated and SIRT1 protein levels were reduced in the
livers of diet-induced obese mice, and FXR activation in these mice reversed the miR-34a and SIRT1 levels, indicating an intriguing link between FXR activation, decreased miR-34a, and subsequently, increased SIRT1 levels. This study demonstrates an unexpected role of the FXR/SHP pathway in controlling SIRT1 levels via miR-34a inhibition and that elevated miR-34a levels in obese mice contribute to decreased SIRT1 levels. Manipulation of this regulatory network may be useful for treating diseases of aging, such as metabolic disease and cancer.

**Introduction**

The NAD$^+$-dependent SIRT1 deacetylase plays a critical role in cellular metabolism, the stress response, and possibly aging, by modulating the activity of its target proteins via protein deacetylation (1-4). SIRT1 (mammalian ortholog of yeast Sir2) is a member of a family of histone deacetylases (HDACs) that regulate chromatin silencing. The founding member of the SIRT1 family was the Silent Information Regulator 2 protein (Sir2) of *Saccharomyces cerevisiae*. The SIRTs are remarkably conserved throughout evolution from *Archaebacteria* to eukaryotes implying that these proteins play vital physiological roles (1-4). SIRT1 in mammals is known to mediate the effect of caloric restriction on longevity by controlling metabolic function and homeostasis and is a key factor in systemic and hepatic glucose, lipid and cholesterol homeostasis. Studies with transgenic (Tg) SIRT1 mice fed a high fat diet showed lower lipid-
induced inflammation along with better glucose tolerance and a mice were almost entirely protected from hepatic steatosis. Downregulation of SIRT1 also decreased serum cholesterol and increased hepatic free fatty acid and cholesterol content which was reversed by overexpression of SIRT1. Recent studies demonstrate that SIRT1 plays an important role in maintaining metabolic homeostasis in response to hormonal and nutritional fluctuations by modulating the activity of PGC-1α, a master metabolic regulator (5,6). During nutritional deprivation, SIRT1 promotes fat mobilization and suppresses adipogenesis and regulates hepatic glucose and lipid metabolism by activating key metabolic regulators, including PGC-1α (5,6).

Activation of SIRT1 by natural or synthetic SIRT1 activators reduced acetylation levels of PGC-1α and protected against diet-induced obesity and insulin resistance by promoting mitochondrial function (7-9). SIRT1 levels are dynamically regulated in response to fasting and feeding under physiological conditions but markedly reduced in the livers of diet-induced obese mice (10). However, the molecular basis by which SIRT1 levels are regulated under normal conditions and why they are substantially reduced in metabolic disease states remains largely unknown.

The nuclear receptor, farnesoid X receptor (FXR), is the primary biosensor for endogenous bile acids and regulates expression of numerous genes involved in lipid and glucose metabolism, in preventing intestinal bacterial infection and gallstone formation and in modulating liver generation and tumorigenesis (11-15). FXR binds to FXR response elements
(FXRE) containing inverted repeats (IR1) or direct repeats (DR4) separated by 4 nucleotides forming a heterodimer with Retinoic X Receptor (RXR, NR2B1). Upon heterodimerization with RXR, FXR binds to the promoter of target genes, such as SHP and increases their transcription.

It is known that FXR indirectly inhibits its metabolic target genes by inducing the expression of small heterodimer partner (SHP), an orphan nuclear receptor and transcriptional corepressor (16,17). It is well established that the FXR/SHP pathway plays an important role in maintaining bile acid and cholesterol levels by inhibiting the transcription of cholesterol 7α hydroxylase (CYP7A1), a key enzyme in hepatic bile acid biosynthesis (16,17) and has been also shown to regulate fatty acid metabolism (18). Interestingly, FXR activation by the synthetic agonist, GW4064, or hepatic overexpression of constitutively active FXR using adenoviral delivery significantly improved hyperglycemia and hyperlipidemia in diabetic obese db/db mice (11). Although both SIRT1 and FXR are critical for hepatic metabolism and activation of both proteins improves metabolic outcomes (7,8,11), it remains largely unknown whether the expression and activity of these two proteins are coordinately regulated.

microRNAs (miRNAs) are recently discovered small (21 to 23 nucleotide) non-coding RNAs that inhibit translation and/or destabilize target mRNAs by binding to their 3’UTRs with partial base pairing (19). In mammals, it originates from a primary transcript, called a pri-miR, which is transcribed by RNA polymerase II and then processed by Drosha and Dicer to become a
mature miRNA (19). miRNAs play important roles in cellular metabolism under normal and metabolic stress conditions (20, 21) and aberrant expression of miRNAs has been observed in human diseases, such as cancer and metabolic disorders (20-23). Here we show that the FXR/SHP regulatory pathway inhibits expression of miR-34a, which results in a positive regulation of hepatic SIRT1 levels under physiological conditions. We further show this regulatory network is altered in diet-induced obese mice, resulting in elevated miR-34a levels and subsequently reduced SIRT1 levels in the liver.

**Materials and Methods**

**Reagents**

Antibodies for FXR (sc-1204, sc-13063), SHP (sc-30169), p53 (sc-6243), HNF-4 (sc-8987), lamin (sc-20680), tubulin (sc-8085), actin (sc-1616), and GFP (sc-8334) were purchased from Santa Cruz Biotech, M2 antibody was from Sigma, and antibodies for SIRT1 and acetylated H3 K9/K14 were purchased from Upstate Biotech.

**Cell Culture**

Human hepatoma cells (HepG2, ATCC HB8065; American Type Culture Collection, Rockville,
MD), mouse hepatoma cells (1c1c7) and monkey kidney cells (COS1) were maintained in phenol-red-free DMEM/F12 (1:1) media. Media were supplemented with 100 U/ml penicillin G-streptomycin sulfate and 10% heat-inactivated fetal bovine serum.

**Construction of adenoviral miR-34a**

For construction of Ad-miR-34a, pri-miR-34a was cloned into the AdEasy-1 plasmid (Stratagene) and recombined homologously with a high frequency in *E. coli* BJ5183. The recombinant plasmids were digested by *PstI* (NEB) and recombinant adenovirus was amplified in AD293 cells and purified following the manufacturer’s instructions (Strategene).

**MicroRNA gene profiling**

Total RNA was isolated from livers from three male wild type mice or FXR null mice using the mirVana TM miRNA isolation kit (Ambion, Austin, TX, USA). Micro RNA microarray studies including labeling, hybridization, image scanning, and data analysis were carried out by Collaborators (Dr. Li Wang’s laboratory in Univ. of Utah) as previously described (24).
**In vivo experiments**

FXR-null mice, ob/ob mice, and congenic CJ57BL mice were purchased from the Jackson laboratory. BALB/c male mice were fed normal chow or high fat western-style chow (Harlan Teklad) for 16-20 weeks. Recombinant adenoviral vectors (0.5-1.0 x10⁹ active viral particles in 200 μl PBS) were injected via the tail vein of mice as previously described (25, 28). A synthetic FXR agonist, GW4064 (30 mg/kg in corn oil), or vehicle were administered by intraperitoneal (IP) injection and mice were sacrificed after 1 hr. Adenoviral-mediated overexpression of flag-FXR was performed as previously described (28). All the animal use and adenoviral protocols were approved by the Institutional Animal Care and Use and Institutional Biosafety Committees at University of Illinois at Urbana-Champaign and were in accordance with National Institutes of Health guidelines.

**Reverse transcriptase (RT) and quantitative RT-PCR**

Total RNA was isolated from mouse liver or HepG2 cells using Trizol reagent, and cDNA was synthesized using a reverse transcriptase kit (Promega). Real-time RT-PCR was performed with an iCycler iQ (Bio-Rad, Inc., Hercules, CA) following the manufacturer’s instructions. The amount of PCR product for each mRNA was normalized by dividing by the amount of β-actin or 36B4 mRNAs. Primers for miRNAs were purchased from Applied
Biosystems. For the RT and qRTPCR of miRNAs, total liver RNA (10 ng) was isolated for the reverse transcription (RT) reaction at 16°C for 30 min and 42°C for 30 min followed by 85°C for 5 min using the Taqman® RT-reaction Kit with Taqman® miR-34a specific primers (Applied Biosystem). Quantitative-PCR was carried out at 95°C for 15 sec (denature) and 60°C for 1 min (anneal/extend) for a total of 40 cycles using iCycler (BioRad). Quantification of expressed miRNAs was normalized with U6 snRNA (Applied Biosystem).

**Antisense miRNAs**

Anti-miR-34a (Ambion®) which is complementary to mature miR-34a sequences was used for down regulation of mature miRNA and anti-N-control (Ambion®) that has a random sequence was used as a control. The anti-miRNAs were modified by conjugation of cholesterol at the 3’-end. From 2.5 to 200 nM of anti-miRNAs was transfected with 1 μl of Lipofectamine 2000 (Invitrogen) in serum free media for 2 days and miRNA levels were analyzed for qRT-PCR.

**CoIP Assays**
Cos-1 cells were grown in six-well plates or 10-cm plates for CoIP assays. Briefly, cells were harvested and resuspended in 250 µl for six-well plates or 500 µl for 10-cm plates of lysis buffer (20 mM K⁺-HEPES (pH 8.0), 0.2 mM EDTA, 5% glycerol, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and protease inhibitors). After incubation on ice for 10 min followed by brief sonication and centrifugation, 1 µg of either control IgG, SHP, or p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the samples were incubated for 4 h to overnight at 4 C. Immune complexes were collected by incubation with 25 µl 25% protein A-Sepharose slurry for 2 h followed by centrifugation. Immunoprecipitates were washed four times with lysis buffer (supplemented with NaCl up to 250 mM). The presence of SHP and p53 in the immunoprecipitates was detected by Western blotting using antisera against SHP and p53.

Transient Transfection and Reporter Assay

HepG2 or Cos-1 cells were cotransfected with luciferase reporter plasmid DNA containing the 3’UTR of SIRT1 or mutated miR-34a binding regions within 3’UTR for the reporter assays with Antisense-miR-34a or Antisense-scrambled negative control as indicated in the figure legends. Twenty-four hours after transfection with Lipofectamine, cells were collected,
and luciferase and β-galactosidase activities were determined. Firefly luciferase activities were
divided by β-galactosidase activities to normalize for transfection efficiency. Consistent results
were observed in at least two independent triplicate transfection assays in each experiment.

**ChIP Assays**

ChIP assays with chromatin isolated from HepG2 cells or in mouse liver were essentially
carried out as previous described. HepG2 cells were infected with Ad-siSHP or Ad-empty as a
control in 15-cm plates (about 1–2 x 10^7 cells per plate) for 2-3 days to knock-down endogenous
SHP. Chromatin was isolated from the cells and incubated with 2–3 µg antibodies or normal
serum. The immune complex was collected by centrifugation and extensively washed, and the
bound chromatin was eluted. Genomic DNA was purified and used as a template for
semiquantitative PCR using primer sets for miR-34a and GAPDH as a control. The amounts of
genomic DNA and PCR cycles used were determined to be within a linear range of amplification.
Each ChIP experiment was repeated two to four times with reproducible results.
Results

FXR down-regulates hepatic miR-34a

FXR is an important regulator of many metabolic genes in liver (13-15) and it is possible that miRNAs, as metabolic regulators, might mediate some FXR actions in hepatocytes. MiRNAs regulated by FXR were detected by miRNA-microarray analysis with hepatic RNA of wild type or FXR null mice (n=3 each group). Of the miRNAs tested, the levels of miR-34a were upregulated the most in FXR null mice. These results were confirmed by q-RTPCR studies in which control miR-720 levels were unchanged and miR-34a levels were elevated about 5-fold although miR-34a levels were not substantially increased in one of four mice which resulted in a large standard error (Fig. 2-1A). These results suggest that FXR usually down-regulates hepatic expression of miR-34a. To test this possibility, normal mice were treated with a synthetic FXR agonist, GW4064. As expected mRNA levels of a known FXR target SHP (16,17) were increased while miR-34a levels were decreased in mice treated with GW4064 (Fig. 2-1B). Similar results were observed in HepG2 cells treated with a natural FXR bile acid agonist, chenodeoxycholic acid (CDCA) (Fig. 2-1C). These results demonstrate that FXR normally down-regulates hepatic expression of miR-34a and, further, raise the possibility that the orphan nuclear receptor and transcriptional corepressor SHP may mediate FXR inhibition of miR-34a gene expression.
SHP mediates FXR inhibition of miR-34a

To determine whether SHP may mediate FXR inhibition of miR-34a gene expression, either siRNA for SHP or exogenous flag-SHP was expressed in livers in vivo by infection with adenoviral vectors. MiR-34a levels were substantially reduced by overexpression of flag-SHP (Fig. 2-1D) but were markedly increased by down-regulation of endogenous SHP with siRNA (Fig. 2-1E). These results strongly suggest that SHP mediates inhibition of miR-34a by FXR in mouse liver in vivo.

SHP interacts with p53, a key activator of the miR-34a gene

The orphan nuclear receptor SHP has been shown to interact with and inhibit numerous DNA binding transcription factors (26,29,30). Since p53 is a key activator of the miR-34a gene (23), we asked whether SHP could inhibit hepatic p53 activity. By CoIP assays, interaction between SHP and p53 in cells overexpressing these proteins was detected. Endogenous SHP also interacted with endogenous p53 in Hepa1c1c7 cells (Fig. 2-2A) and in liver extracts (Fig. 2-2B). In in vitro GST-pull down assays, SHP interacted with GST-p53, whereas FXR did not (Fig. 2-2C). These results indicate that SHP directly interacts with p53 in hepatic cells and mouse liver.
SHP inhibits p53 transactivation of the miR-34a promoter

To test whether SHP inhibits p53 transactivation ability on the miR-34a promoter, we next examined the effects of down-regulation of SHP on miR-34a promoter activity. SHP mRNA and protein levels were efficiently decreased in cells infected with Ad-siSHP (Fig. 2-2D), and miR-34a promoter activity was increased in a dose-dependent manner, but not by infection with control Ad-empty (Fig. 2-2E). Further, mutation of the p53 binding site nearly abolished the promoter activity (Fig. 2-2F, lanes 2, 5) indicating that p53 is the dominant regulator of miR-34a expression. These results, taken together, indicate that SHP inhibits transactivation of p53, a key activator of miR-34a promoter activity. In addition, although activity was nearly abolished by mutation of the p53 binding site, small increases were observed in cells expressing SHP siRNA (Fig. 2-2F, lanes 5, 6) compared with wild type cells (lanes 2, 3) which suggests that SHP may also interact with and inhibit transcription factor(s) other than p53 at the miR-34a promoter. A potential DNA binding site for HNF-4, a known SHP-interacting protein (30), is present at upstream of the p53 binding site in the miR-34a promoter. Overexpression of HNF-4, but not other nuclear receptors including FXR, enhanced p53-transactivation of the miR-34a promoter activity. Similar transactivation of the miR-34a promoter by HNF-4 was observed with a promoter lacking the p53 binding site, indicating that HNF-4 is a minor contributor to miR-34a activation in a p53-independent manner.
**SHP inhibits p53 binding to the miR-34a promoter**

To delineate mechanisms by which SHP inhibits p53-transactivation of miR-34a, we performed chromatin immunoprecipitation (ChIP) assays. Occupancy of p53 was substantially decreased while that of SHP was increased after GW4064 treatment (Fig. 2-2G) and similar results were observed in HepG2 cells (Fig. 2-2H). These results suggest that activated FXR signaling results in dissociation of p53 and recruitment of SHP at the miR-34a promoter. To directly determine whether SHP is involved in decreased occupancy of p53 at the miR-34a promoter, SHP was down-regulated with siRNA. The inhibition of p53 binding to the miR-34a promoter by GW4064 treatment was markedly reversed by expression of SHP siRNA, while little binding to the control GAPDH gene was observed (Fig. 2-2I). These results demonstrate SHP dependent inhibition of p53 binding to the miR-34a promoter. Since SHP has been shown to directly interact with HNF-4 (30), we also examined whether HNF-4 plays a role in the recruitment of SHP to the miR-34a promoter. Occupancy of HNF-4 at the miR-34a promoter was decreased in cells expressing siRNA for HNF-4 as expected and the association of SHP at the promoter was decreased. These results suggest that HNF-4 may also play a role in the recruitment of SHP to the miR-34a promoter.
miR-34a binds to the 3’ UTR of SIRT1 transcript

miR-34a was shown to regulate cell proliferation and apoptosis by imperfect base-pairing to the 3’UTR of SIRT1 mRNA (Fig. 2-3A) in human colon cancer cells (22). To test whether miR-34a inhibits SIRT1 activity in hepatic cells, reporter assays were performed using gain- and loss-of-function experiments. Overexpression of miR-34a decreased the 3-UTR-SIRT1-luc activity in a dose-dependent manner (Fig. 2-3B, left panel) and these effects were blunted in 3’UTR SIRT1-luc in which miR-34a binding sites are mutated (Fig. 2-3B, right panel). Overexpression of anti-miR-34a significantly increased the reporter activity (Fig. 2-3C, lanes 1, 3), whereas control scrambled RNA did not (lanes, 1 and 4). These results confirm the previous findings that SIRT1 is a target of miR-34a (22) and show that this inhibition occurs in hepatic cells.

miR-34a down-regulates hepatic SIRT1 protein levels

Exogenous expression of miR-34a also significantly reduced SIRT1 protein levels in a dose-dependent manner, but SIRT1 mRNA levels were only modestly, and not statistically significantly, reduced (Fig. 2-3D). These results indicate that miR-34a downregulates SIRT1 protein levels in hepatic cells. In in vivo studies, infection of mice with either Ad-miR-34a or control Ad-empty resulted in similar infection efficiency, detected by GFP expression, and
elevated miR-34a levels in mice infected with Ad-miR-34a. Consistent with the HepG2 cell
studies, overexpression of miR-34a in mouse liver significantly decreased SIRT1 protein levels
(Fig. 2-3E). These results demonstrate that SIRT1 is a target of miR-34a in mouse liver in vivo.

Elevated hepatic miR-34a and reduced SIRT1 levels in FXR null mice

The results above suggest that activation of a regulatory pathway involving FXR and
SHP inhibits miR-34a expression resulting in increased SIRT1 levels. Because miR-34a levels
were highly elevated in FXR null mice (Fig. 2-1) and miR-34a targets SIRT1 (Fig. 2-3), we next
asked whether SIRT1 levels are decreased in FXR null mice. Consistent with the results that
miR-34a levels were increased in FXR-null mice (Fig. 2-1A), SIRT1 protein levels were indeed
substantially decreased (Fig. 2-4A), whereas SIRT1 mRNA levels were not changed (Fig. 2-4B).

Deregulation of hepatic miR-34a and SIRT1 levels in high fat diet-induced obese mice

FXR-null mice exhibit pro-atherogenic lipid profiles and elevated lipid and glucose
levels (11,13,31) and activation of FXR in metabolic disease mice resulted in improved
metabolic outcomes (11). Therefore, to examine whether the FXR targets miR-34a and whether
SIRT1 levels are altered in metabolic disease, we utilized high fat westernstyle diet (WD)-
induced obese mice (Fig. 2-4C). Hepatic lipid levels and mRNA levels of lipogenic FAS and CD36 genes were substantially elevated in WD mice (Fig. 2-4D, E). Interestingly, miR-34a levels were significantly elevated both in diet-induced obese mice (Fig. 2-4F) and in leptin-deficient ob/ob mice (Fig. 2-4G). Consistent with increased miR-34a expression in diet-induced obese mice (Fig. 2-4F), association of RNA polymerase II, detected by ChIP assay, was increased at the miR-34a promoter and acetylated histone H3K9/K14, a gene activation histone code (32), was dramatically elevated (Fig. 2-4H). Consistent with elevated miR-34a levels, reduced SIRT1 protein, but not mRNA, levels were detected in liver (Fig. 2-4I, J).

**FXR activation decreases hepatic miR-34a levels and increases SIRT1 levels in obese mice**

It was demonstrated that activation of FXR in db/db mice improved metabolic profiles by decreasing serum glucose and lipid levels (11). We, therefore, asked whether activation of FXR in diet-induced obese mice could alter miR-34a levels and subsequently, SIRT1 protein levels in these mice (Fig. 2-5A). Treatment with the FXR activator, GW4064, daily for 5 days substantially reduced the miR-34a levels in diet-induced obese mice compared to those in normal mice (Fig. 2-5B). Remarkably, hepatic SIRT1 protein levels were substantially increased in obese mice treated with GW4064 (Fig. 2-5C). In control experiments, treatment with GW4064 increased mRNA levels of Shp and decreased mRNA and protein levels of p53 in diet-induced
obese mice. Overexpression of FXR in mouse liver using tail vein injection of Ad-3flag-FXR also resulted in substantial increases in SIRT1 protein levels in diet-induced obese mice (Fig. 2-5D). These results demonstrate an intriguing link between FXR activation, decreased miR-34a levels, and subsequently, increased hepatic SIRT1 protein levels in obese mice.

**Discussion**

Despite recent advances in understanding the biological functions of SIRT1, the molecular basis by which SIRT1 levels are regulated under normal conditions and why they are substantially reduced in metabolic disease states remains unclear. Our studies, at least in part, provide an explanation by showing that the FXR/SHP pathway plays a role in controlling hepatic SIRT1 levels via miR-34a inhibition and that elevated miR-34a levels in obese mice contribute to decreased SIRT1 protein levels. As summarized in Fig. 2-5E, in normal mice, hepatic miR-34a levels are regulated via a cascade pathway involving the nuclear bile acid receptor FXR and an orphan nuclear receptor and transcription corepressor SHP. Activation of FXR signaling inhibits the expression of the hepatic miR-34a gene through the induction of SHP. SHP suppresses transcription of the miR-34a gene by inhibiting the promoter occupancy of p53, the key activator of the miR-34a gene. In contrast, in diet-induced obese mice, the FXR/SHP pathway is deregulated so that miR-34a levels are highly elevated, which contributes to reduced
SIRT1 protein levels. Remarkably, activation of FXR in these obese mice by daily treatment with GW4064 for 5 days restores SIRT1 levels by decreasing miR-34a levels. Edwards and colleagues previously demonstrated that daily treatment of diabetic obese db/db mice with GW4064 for 5 days improved metabolic profiles by decreasing serum glucose and lipid levels (11). Therefore, our findings, together with these previous studies, indicate an intriguing loop of positively interacting FXR and SIRT1 regulation.

Interestingly, SIRT1 may positively regulate hepatic FXR as well. PGC-1α was shown to enhance FXR activity in the regulation of triglyceride metabolism during fasting by increasing expression of the FXR gene and also by coactivating FXR transactivation (37). Since SIRT1 deacetylates and increases PGC-1α activity (1,6), SIRT1 should increase FXR activity via modulation of PGC-1α activity. Further, we recently reported that SIRT1 increases FXR transactivation ability by dynamic deacetylation of FXR (28). FXR acetylation inhibits its transactivation potential by inhibiting interaction of FXR with RXR and subsequently DNA binding (28). While FXR acetylation is dynamically modulated by p300 and SIRT1, FXR acetylation levels are highly elevated in ob/ob mice and diet-induced obese mice (28). These previous studies, along with current findings, suggest that an intriguing positive regulatory loop between FXR and SIRT1 is operating in hepatocytes in normal conditions.
In obese mice, however, the FXR/SHP signaling may be deregulated due to highly elevated FXR acetylation levels, which results in elevated miR-34a levels. In accordance with important roles of FXR in regulating miR-34a and SIRT1 protein levels, miR-34 levels were elevated and SIRT1 protein levels are substantially decreased in FXR null mice. Staels and his colleagues demonstrated that FXR plays an important role in adaptive responses during the transition from feeding to fasting and fasting associated responses were markedly blunted in FXR null mice (38,39). In line with these results, Auwerx and his colleagues demonstrated that SIRT1 levels are dynamically regulated under fasting and feeding in normal mice (10). SIRT1 levels were modestly increased upon short-term fasting (6 h) but prominent expression was still observed in the fed state in normal mice (10). We, therefore, envision that under normal conditions, FXR dynamically regulates metabolic pathways in response to fasting and feeding cycles and activated FXR signaling during feeding would contribute to maintenance of basal levels of hepatic SIRT1 via miR-34a inhibition, which would allow for an efficient transition to the next fasting cycle.

In contrast, this regulatory network is altered in metabolic disease states, resulting in constitutively elevated miR-34a levels and subsequently reduced SIRT1 levels in the liver. Recent studies demonstrate that SIRT1 deacetylates and inhibits p53 activity, which results in decreased expression of miR-34a (3,22,33,34). Consistent with this feedback regulatory loop, we
also observed that overexpression of SIRT1 in liver of diet-induced obese mice using tail vein injection of Ad-flag-SIRT1 substantially decreased hepatic miR-34a levels. Furthermore, daily treatment for 1 week with resveratrol, which activates SIRT1, also decreased miR-34a levels in obese mice. Resveratrol indirectly activates SIRT1 by AMPK activation (35,36) so the possibility that resveratrol is acting in a SIRT1-independent manner cannot be ruled out.

However, the SIRT1 expression studies, combined with the resveratrol results, further support the existence of the regulatory loop between miR-34a and SIRT1 in hepatocytes in which SIRT1 inhibits expression of its own inhibitor, miR-34a, thereby, further enhancing SIRT1 expression.

miRNAs are emerging as important cellular regulators critically involved in diverse biological pathways including metabolic regulation, cell proliferation, and apoptosis (19,34). Approximately 30% of all human genes are thought to be regulated by miRNAs and elevated levels of miRNAs have been detected in diverse pathophysiological conditions (20,21). Recent in vivo studies demonstrated that antisense miRNAs may have therapeutic value by down-regulating miRNAs in disease conditions (20,40). It will be important to see whether down-regulation of elevated miR-34a in obese mouse liver using anti-miR34a approaches would increase hepatic SIRT1 levels and improve metabolic outcomes in these mice. Modulation of SIRT1 levels by manipulation of the FXR-miR34a-SIRT1 pathway, therefore, may provide a
novel therapeutic target for treating aging-related diseases, such as metabolic disorders and cancer, in which SIRT1 plays an important role.
Fig. 2-1

A

miR-34a

miR-720

FXR+/+
FXR-/-

Rel miR level

B

mouse SHP

Rel mRNA level

Veh GW4064

C

human SHP

Rel mRNA level

Veh CDCA

D

miR-34a

Ad-E Ad-f-SHP

Rel miR level

E

miR-34a

PBS Ad-E Ad-f-SHP

WB: SHP

0.4 0.3 0.2 0.1

Ad-E + -

Ad-siSHP + ++
Fig. 2-1. FXR down-regulates miR-34a.

A, hepatic miR-34a and control miR-720 levels from wild type (+/+ ) and FXR-null mice (-/- ) were measured by qRT-PCR, and values from each individual mouse were plotted. Bars indicate average values from three or four animals. B, normal mice were treated with GW4064 or vehicle (Veh) for 1 h, and livers were collected for qRT-PCR, and values from each individual mouse were plotted. Bars indicate average values from five to seven animals. C, HepG2 cells were treated with 50 M chenodeoxycholic acid (CDCA) overnight and collected for qRT-PCR. Error bars, Standard Deviation. D and E, normal mice were injected via the tail vein with adenoviral vectors as indicated, and 5 days later, hepatic miR-34a levels were measured by qRT-PCR and SHP protein levels were measured by Western blot (WB) analysis. Statistical significance was measured using Student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; NS, statistically not significant. (Lee et al. 2010)
Fig. 2-2

A. CoIP: Hepa 1c cells

B. CoIP: Mouse liver

C. GST pull down

D. SHP mRNA level

E. miR34a-luc

F. miR34a-luc (-p33 site)-luc

G. mouse liver

H. HepG2

I. HepG2 cells
Fig. 2-2. SHP inhibits expression of the miR-34a gene by inhibiting p53 binding to the promoter. A and B, CoIP assays were done using mouse Hepa1c1c7 cell (A) and mouse liver (B) extracts to detect interaction between endogenous p53 and SHP. C, 35S-SHP or 35S-FXR was synthesized, and binding to GST or GST-p53 was determined by GST pulldown assays. D–F, HepG2 cells were cotransfected with expression plasmids as indicated and with the miR-34a promoter (1402 to 578)-luc or a mutant reporter and then infected with Ad-siSHP. Two days later, cells were collected for qRT-PCR (D) and reporter assays (E and F). Standard Deviation (S.D). G and H, mouse (G) or HepG2 cells (H) were treated with 100 nM GW4064 for 1 h, and samples were collected for ChIP assays. Band intensities were measured, and the intensities relative to samples treated with vehicle are indicated at the top of each band. I, HepG2 cells infected as indicated were treated with GW4064 and collected for ChIP assays. (Lee et al.2010)
Fig. 2-3

A

3'UTR-SIRT1

miR-34a

AACACCCAGCUAGGACCAUUAUGCCAG

UGUUGGUCGAUUCUG----UGACGGU--

(Adapted from Yamakuchi et al., 2008)

B

3'-UTR-SIRT1-luc

3'-UTR-mut-SIRT1-luc

C

3'-UTR-SIRT1-luc

D

SIRT1

Tubulin

E

SIRT1 protein levels

SIRT1 protein levels

Ad-miR-34a

Ad-E

Ad-miR-34a
Fig. 2-3. SIRT1 is a target of miR-34a in hepatocytes.
A, the miR-34a sequence and miR-34a binding site in the 3-UTR of the SIRT1 transcript are shown. B, HepG2 cells were transfected with plasmids as indicated and then infected with Ad-miR-34a or control Ad-empty and collected for reporter assays. C, COS-1 cells were transfected with reporter plasmids as indicated along with anti-miR-34a or control scrambled RNA (Anti-scrm) and then collected for reporter assays. D, HepG2 cells were infected with Ad-miR-34a or control Ad-empty, and SIRT1 and control tubulin levels were detected. E, normal mice were injected in the tail vein with Ad-miR-34a or control Ad-empty, and 5 days later, hepatic SIRT1 and control actin and tubulin levels were measured. In the lower panels of D and E, band intensities were measured, and the intensities relative to those of tubulin were plotted. Statistical significance was determined by the Student’s t test (Standard Deviation., n=3). **, p<0.01; ***, p<0.001. (Lee et al. 2010)
Fig. 2-4. Elevated miR-34a and decreased SIRT1 levels in diet-induced obese mice.

A and B, hepatic SIRT1 protein levels from wild type or FXR-null mice were detected by Western blot analysis (A) and qRT-PCR (B). A, right panel, band intensities relative to those of tubulin were plotted. Statistical significance was determined by the Student’s $t$ test (Standard Deviation., $n=4$). *, $p<0.05$; NS, statistically not significant. C–J, C, experimental outline. Livers from mice fed normal chow (ND) or a high fat Western-style diet (WD) for 20 weeks were collected for oil red o staining (D), qRT-PCR (E, F, G, and J), ChIP assays (H), and Western blot analysis (I). I, band intensities relative to those of tubulin were plotted. Statistical significance was determined by the Student’s $t$ test (Standard Deviation, $n=5$). *, $p < 0.05$. (Lee et al. 2010)
Fig. 2-5

A

Western Diet (20 weeks) → Daily treatment with GW4064 (B, C) for 5 days → Ad-f-FXR (1 week) → CA feeding (D)

1. q-RTPCR (miR-34a levels)
2. Western analysis (SIRT1 levels)

B

miR-34a

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C

Animal set

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D

SIRT1

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E

Normal physiology

Metabolic disease states

- Deregulated FXR/SHP activity
- Elevated miR-34a levels
- Decreased SIRT1 levels
Fig. 2-5. Activation of FXR decreased miR-34a levels and increased SIRT1 protein levels in diet-induced obese mice.

A, experimental outline is shown. B and C, mice fed WD chow were treated daily with GW4064 for 5 days, and hepatic miR-34a levels (B) and SIRT1 protein levels (C) were measured. D, mice fed WD chow were injected with Ad-FLAG-FXR, and 1 week later, mice were fed 0.5% cholic acid-chow for 6 h; hepatic SIRT1 protein levels were measured. E, a proposed model for FXR/SHP/miR-34a regulatory network controlling hepatic SIRT1 levels is shown. In this model, miR-34a inhibits translation of hepatic SIRT1 by binding to the 3-UTR of SIRT1 transcript with partial base pairing. In normal mice, activation of FXR induces expression of SHP, which suppresses transcription of the miR-34a gene by inhibiting p53 binding to the miR-34a promoter. In contrast, in diet-induced obese mice, the FXR/SHP pathway is defective, which results in elevated miR-34a levels and subsequently decreased SIRT1 protein levels in the liver. (Lee et al. 2010)
Fig. 2-6

Fig. 2-6. The FXR/SHP pathway controlling miR-34a and SIRT1 expression.
Under normal conditions, activation of FXR signaling induces the metabolic repressor SHP in liver. SHP is then recruited to the miR-34a promoter and inhibits binding of the key activator p53 to the DNA, resulting in decreased miR-34a expression. Inhibition of miR-34a results in increased hepatic SIRT1 levels. In contrast, under pathophysiological conditions such as fatty livers of obese mice, the dysregulated FXR/SHP pathway due to highly elevated FXR acetylation no longer inhibits transcription of miR-34a. The dysregulated FXR/SHP pathway, along with acetylation of p53 due to cellular stress under metabolic disease states, results in elevated miR-34a expression, which contributes to decreased SIRT1 levels.
(Lee and Kemper, 2010)
References


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Chapter Three

Genome-wide analysis of hepatic FXR bindings
in normal and diet-induced obese mice

Abstract

FXR is a ligand-dependant transcriptional factor that regulates glucose homeostasis and lipid biogenesis, as well as bile acid biosynthesis, in liver by binding to a DNA response element (FXRE) as a heterodimer complex with RXRα or as a monomer. In metabolic disease, FXR activation by treatment with synthetic or natural ligands improves symptoms like hyperlipidemia and hyperglycemia. Although hepatic FXR binding sites after activation of FXR in the whole genome have been described, it remains unknown whether FXR binding to its target genes in disease states, like fatty liver induced by a high fat diet, occurs at genomic locations similar or different from normal animals. Here, we identify genomic FXR binding sites in fatty livers induced by high fat diet (HFD) using chromatin immunoprecipitation (ChIP) followed by high throughput DNA sequencing (ChIP-seq). There were 15,263 or 5,272 FXR binding sites detected in the livers of ND or HFD fed mice, respectively, after GW4064 treatment. Most of the sites are located in intergenic and intronic regions at IR-1 motifs in both groups. Interestingly, 7,440 of the FXR binding sites detected only in normal liver included groups of genes encoding Ca$^{2+}$/K$^+$
channels, S/T kinases, and lipid oxidation enzymes, while 2,344 of the FXR binding sites detected only in fatty livers included iron-sulfur binding proteins, Toll like receptor and oncogenic proteins. Analysis of the mRNA levels of the selected genes containing unique FXR binding sites in either ND or HFD mice demonstrated FXR dependent regulation of the mRNA levels indicating that binding sites are functionally significant and thus FXR binding correlates with different biological functions in normal and metabolic diseases mice.

**Introduction**

Farnesoid X Receptor (FXR) is a bile acid-activated transcription factor that controls the expression of genes responsible for diverse functions like bile acid biosynthesis, glucose and lipid homeostasis, liver regeneration, pro-inflammation and tumorigenesis (1-3). It regulates the expression of numerous metabolic genes and, thus, is a crucial player in liver metabolism (3,4). The activation of FXR by an agonist results in reduced plasma triglyceride, hepatic lipogenesis, cholesterol levels, synthesis of free fatty acids (FFAs), and the secretion of VLDL from liver (5). Increased LDL and induction of genes involved in lipoprotein metabolism after FXR activation is consistent with previous findings of its effects on plasma lipids (3-5). In addition, FXR exerts an important role in glucose homeostasis. FXR deficiency in mice leads to impaired glucose tolerance and insulin resistance (6, 7). Activation of hepatic FXR regulates gluconeogenesis,
glycogen synthesis and insulin sensitivity. It was shown that treatment of mice with the FXR agonist, GW4064 or cholic acid (CA), or infection with adenovirus that expresses a constitutively active FXR-VP16 fusion protein, resulted in a significant reduction of plasma glucose levels and improved insulin sensitivity. Disruption of the FXR gene in transgenic mice was associated with metabolic diseases including hypercholesterolemia, hyperglycemia, cholesterol gallstone disease, fatty liver, and type II diabetes (6).

Since the mouse genome has been sequenced, it is possible, in principle, to determine how many sites for a given transcription factor are present across the entire genome for a given cell type or physiological condition (8). Such measurements are important to provide a global map of gene networks with input connections. These direct physical interactions between transcription factors and the chromosome can be detected by chromatin immunoprecipitation (ChIP). In ChIP experiments for DNA-binding proteins, chromatin DNA fragments associated with a specific protein are enriched (9). The DNA-binding proteins are crosslinked to chromatin DNA in vivo by treating cells with formaldehyde and the chromatin is sheared by sonication into small fragments, which are generally in the 200-600 bp range. An antibody specific to the protein of interest is used to precipitate the DNA-protein complex. Finally, the crosslinks are reversed and the released enriched DNA sequences are then purified and in global genomic analyses are quantified by microarray (ChIP-chip) or by direct ultra high-throughput DNA
sequencing (ChIP-seq) (10, 11). Owing to the rapid technological developments in next-generation sequencing (NGS), the ability to sequence tens or hundreds of millions of short DNA fragments in a single run enables a global analysis. In ChIP-seq, the DNA fragments of interest are sequenced directly instead of being hybridized on an array. This method has higher resolution, fewer artefacts, greater coverage and a larger dynamic range than ChIP-chip and therefore provides substantially improved data (12). The more precise mapping of protein-binding sites provided by ChIP-seq allows for a more accurate list of targets for transcription factors and enhancers, in addition to better identification of sequence motifs.

Recently, the genome-wide map of FXR binding sites in liver and intestine after activation of FXR with GW4064 has been elucidated using ChIP-seq (13). As a ligand-dependant transcriptional factor, FXR is known to activate gene expression by binding to FXREs within the promoters of target genes including Shp as a heterodimer with RXRα in response to bile acid (2). Recently, it was reported that FXR suppresses Apolipoprotein (ApoAI) transcription via a negative FXR response element in humans (14). After GW4064 treatment, 1,656 binding sites containing an identifiable IR-1 site were detected for FXR in the liver (13). Most of the sites were located primarily in intergenic and intronic regions but there was a significant enrichment of FXR-binding sites within 2 kb of transcription start sites (TSS) for known genes. Motif analysis revealed a half nuclear receptor binding site, normally bound by a few orphan nuclear
receptors, adjacent to the FXR response elements, indicating possible involvement of some orphan nuclear receptors in modulating FXR function (15). Despite the importance of the FXR-mediated genomic cistrome in liver, a complete understanding of FXR-DNA interaction at the genomic level especially in disease states is not known.

Interestingly, our group found constitutively elevated levels of FXR, acetylated at K217 in the livers of genetic obese mice (ob/ob) and diet-induced obese mice (16). Acetylated FXR impaired the interaction with RXRα and binding to the FXRE (16). These data imply that FXR might differentially regulate its target genes in metabolic disease due to the post-translational modification that may alter DNA binding, cofactor interaction or protein stability. While recent studies provide great insight into the binding sites to which FXR binds in response to bile acids in normal animals, binding of FXR in metabolic disease conditions, like high fat diet-induced obesity, may be different. Therefore, we examined genome-wide chromatin localization of FXR using ChIP-seq analysis in the livers of mice fed ND or HFD. By comparison of binding sites of FXR in normal and the diseased liver induced by diet, we are able to evaluate the role of FXR regulation in the healthy liver and the effects of misregulation of FXR on pathophysiology or related gene regulation in the metabolic disease state. Furthermore, understanding how FXR function is dysregulated in disease states may lead to novel therapeutic agents for treating age-related metabolic diseases, including fatty liver, obesity and type II diabetes.
Materials and Methods

Animals

Balb/c male mice were fed with normal chow as a control group or high fat chow (42% fat, Tek Harlan) at least 3 to 4 months to induce a metabolic disease state, like fatty liver (17). A synthetic FXR ligand GW4064 (30 mg/kg/day) or vehicle (DMSO) in corn oil (Sigma) was injected intraperitoneally for 1 h. Mouse livers were removed and used for the ChIP assays. The hepatic mRNA levels of Shp were measured by qRT-PCR to monitor activation of FXR by GW4064. For gene expression assays, mice were treated with GW4064 by i.p. injection overnight for qRT-PCR.

Chromatin Immunoprecipitation (ChIP)

Liver tissues from mice were collected and proteins and DNA were cross-linked with 1% formaldehyde in PBS at room temperature for 10 min. Cross-linking was stopped by adding 125 mM glycine followed by washing the samples twice with ice-cold PBS. The tissues were resuspended in hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.2% NP40, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 5% sucrose) and cells were broken by Dounce homogenization and nuclei were isolated by centrifugation. The nuclei were
resuspended in sonication buffer (50 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% SDS) and
sonicated to reduce the DNA length to 200-800 bp. After centrifugation, the supernatant was
diluted, pre-cleared by incubation with 25% protein G-Sepharose at 4 °C for 30 min, and
chromatin was immunoprecipitated with 2-3 µg of antibodies to FXR (Santa Cruz, H130 and
C20), RXR (Santa Cruz, D20), RNAP II (Santa Cruz, H224), or IgG as a negative control, at 4 °C
overnight. The immune complexes were collected by incubation with 50 µl of a 25% protein G
Sepharose slurry for 1 h and the beads were washed extensively with low salt, high salt, and LiCl
wash buffers followed by wash twice with Tris-EDTA (TE) buffer as described previously (17).
After the elution of bound chromatin, the genomic DNA was purified by phenol-chloroform
extraction and subjected to PCR or qPCR using primers (Table. 3-6) specific for the *Shp* and
*Ostβ* promoter region which are known to contain a FXR binding region or *Gapdh* promoter as a
negative control (13). For Re-ChIP assays, chromatin immunoprecipitated with FXR antibody
(c20, Santa Cruz) was eluted by adding 10 mM DTT at 37 °C for 30 min. The eluted components
were then diluted with dilution buffer (20 mM Tris-HCl pH 8.1, 150 mM NaCl, 2mM EDTA,
1% Triton X-100) followed by reimmunoprecipitation with the second antibodies (FXR, FXR-
Acetylated K217, RXR, RNA Pol II, or IgG) for re-ChIP. After overnight incubation with
antibodies, precipitated chromatin was then analyzed in the same way as for standard ChIP
assays.
High throughput sequencing of ChIP-DNAs and genomic mapping of DNA reads

Twenty ng of ChIP-DNA which was immunoprecipitated by FXR antibody, or IgG (negative control) in both groups were used for ChIP-seq library construction (Illumina). Deep sequencing was then done using the Illumina/Solexa Genome Analyzer II (Biotechnology Center, UIUC). Fully sequenced ChIP-DNA (50 bp) was used for genome alignment with *Mus musculus* genome sequences, NCBI m37 genome assembly (mm9: July 2007) as a reference sequence, allowing 2 bp mismatches. FXR binding DNA peaks aligned to mouse genome sequences were subjected to CisGenome (v1.2) to generate peaks with peak threshold using a false discovery rate (FDR) cutoff (<0.01 or <0.001). For the FXR peak generation in the GW4064-treated samples, two criteria such as FDR (<0.01) and ratio (>5) of FXR binding to IgG peaks were used in both diet groups to detect binding sites (18).

Annotation and Gene Ontology (GO) analysis

FXR binding sites (15,263 and 5,272) in ND or HFD samples were analyzed to identify the gene locations of the sites in the mouse genome. A list of all genes whose nearest FXR peaks were within ± 10 kb of known genes as generated using CisGenome (19). Annotated genes near FXR binding sites uniquely detected in either the ND or HFD groups were 1,223 or 354,
respectively. GO analysis of FXR target genes was conducted by using the NIH database for Annotation, Visualization, and Integrated Discovery (DAVID: http://david.acc.ncifcrf.gov/) for functional grouping of binding genes (20). This analysis was also used to classify the genes into functionally related groups by using PANTHER biological process terms (http://www.pantherdb.org). All sequence analyses were conducted based on the Mus musculus NCBI m37 genome assembly (mm9:July 2007) accessed from Ensemble. Enrichment scores and P-values were also generated by this analysis.

Motif search

The enrichment of motifs within the 250 top scoring FXR binding peaks with exception of simple repeats in the ChIP-seq data of ND or HFD fed groups was analyzed in MEME (Multiple Em for Motif Elicitation; http://www.meme.sdsc.edu) as position dependent letter-probability matrices (21, 22). The coordinates of each peak are set to collect motif lengths of 6 to 20 bp. Comparison of motifs against a database of known motifs was done in TOMTOM generating p-values of the similarity score, scoring details and a logo alignment for each match.

Validation of FXR binding sites by ChIP-qPCR
Independently performed ChIP assays were analyzed by qPCR to validate genes identified by ChIP-seq with power SYBR green PCR master mix (Taqman) and primers in Real time PCR system (StepOnePlus, Applied biosystem) listed in Table.3-6. ChIP-qPCR data analysis used a normalization method of fold enrichment by comparison with FXR non-binding genes like mouse $\beta$-actin or mouse 36B4 or IgG binding DNAs.

**Quantitative RT (qRT)-PCR**

Total RNA from the livers of mice treated with GW4064 overnight was isolated using Trizol (Invitrogen). Reverse Transcriptase (RT) reactions were performed as described in Chapter II. QRT-PCR (StepOne Plus, Applied Biosystems) was performed using primers listed in the Table. 3-5. the results of qRT-PCR are normalized for all genes of interest to a house keeping control gene $\beta$-actin, which does not significantly vary in normal and metabolic disease (17).
**Results**

**ChIP-PCR and -qPCR**

Balb/c male mice fed high fat diet chow (HFD) *ad libitum* at least 3 months had body weights significantly increased by 18% when compared to normal diet (ND) mice. Profiles including the date of birth and body weight of mice are shown in Table 3-1. Expression of lipogenic genes including *Scd1* and *Srebp-1c* were increased consistent with fatty liver in the HFD group compared to the ND group (Fig. 3-1A). Then mice in both diet groups were injected intraperitoneally with GW4064 (30 mg/kg body weight/day) to activate FXR or vehicle (10% DMSO) as a control. After 1 h, mRNA levels of *Shp* were monitored by qRT-PCR to monitor whether GW4064 treatment effectively activated FXR in the normal liver (Fig. 3-1B). Then, livers from the mice of both diet groups were subjected to ChIP assays using antibodies to FXR, RXR or no antibody (IgG, mock IP). ChIP-PCR and –qPCR showed measurable and reproducible recruitment of endogenous FXR to the known FXREs such as *Nr0b2 (Shp)* or *Ostβ* genes in the normal liver indicating that the ChIP assay was performed successfully (Fig. 3-2) (13). Additionally, recruitment of the heterodimer partner of FXR, RXRα, at the same FXR binding sites was detected as a positive control in both diet groups (Fig. 3-2B). ChIP-DNAs (50
ng/sample) using 4 independently performed ChIP experiments were then analyzed by deep sequencing (Biotechnology Center, UIUC).

**Genomic mapping for FXR binding in liver**

High throughput massive DNA sequencing generated around 3-6 million ChIP-DNA reads for the ChIP-seq analysis for each sample (Table. 3-2). All the DNA reads (50 bp in length) were aligned for genomic mapping using mouse genome (NCBI37: mm9) as a reference sequence allowing 2 bp mismatches. The mapping rate for FXR binding DNAs (Number of DNA reads mapped over total number of DNA reads sequenced) was 54.2% for ND mice with vehicle, 49.5% for ND mice with GW4064, 64.7% for HFD mice with vehicle (6,051,048/9,346,168) and 55.5% for HFD mice with GW4064 with an FDR of < 0.01 (Table. 3-2). For the negative control, 48.2% of non-specific binding peaks generated with IgG were mapped.

**Identification of activated FXR binding sites in the livers of mice fed ND or HFD**

The fragments of DNA reads at each site after genomic mapping using total DNA reads was analyzed to generate FXR binding peaks after FXR activation. A one sample analysis in CisGenome eliminated non-specific small FXR binding peaks using a threshold which is
indicated as the minimum size of FXR peaks with an FDR of < 0.01 (Table 3-3), thereby generating 39,234 FXR binding sites in normal livers. After GW4064 treatment, 71,434 FXR binding sites were detected with same criteria, which is an increase compared to that with vehicle and in agreement with a previous report (13).

In order to identify FXR binding sites with high confidence after FXR activation, a two sample analysis was performed in CisGenome with an FDR of <0.01 and non specific IgG binding peaks with a ratio of FXR to IgG peaks of <5 were removed (18). In normal liver, 15,263 of FXR binding site were identified that had higher peaks in GW4064 treated samples than in vehicle suggesting these peaks represent FXR binding sites after FXR activation (Fig. 3-3). In order to illustrate FXR binding sites after GW4064 activation, representative FXR binding peaks with vehicle or GW4064 treatment in ND fed mice that were displayed on the UCSC genome browser as shown in Fig. 3-4. Well known FXR target like the promoter regions of Shp (Nr0b2) and Ostβ in liver that contain FXREs (IR-1) was also detected in our ChIP-seq data providing confidence that our ChIP-seq data are detecting valid FXR target genes. In addition, novel FXR binding sites near genes like Cyp8b1 and Tnfsf4 were also identified by our ChIP-seq data, suggesting that these genes might be regulated in the liver by FXR for bile acid homeostasis for Cyp8b1 and inflammation for Tnfsf4.
After identifying FXR binding peaks, 15 randomly selected FXR binding sites in ND were subjected to ChIP-qPCR to validate FXR binding in the ChIP-seq results. Thirteen out of 15 sites (87%) revealed FXR recruitment on the selected loci (Fig. 3-5). ChIP-DNAs pulled from 3 independent ChIP assays were adapted for qPCR analysis. One of the well known FXR binding sites containing an IR-1 FXR binding motif, the \textit{Nr0b2} region which encodes SHP, was analyzed in 3 independent ChIP-qPCR assays showed consistent FXR recruitment on the promoter region of Shp.

Likewise, 75,862 FXR binding peaks with vehicle in fatty livers induced by HFD were identified, while only 22,931 were detected with GW4064 treatment (Table 3-3). Two sample-analysis using these FXR binding peaks generated 5,272 FXR binding sites which indicate FXR binding sites after FXR activation in the HFD group (Fig.3-2). FXR binding sites in the HFD group were visualized on the UCSC genome browser and FXR binding sites on the selected sites and illustrated in Fig. 3-6. Novel metabolism genes with FXR binding sites like \textit{Adh7}, \textit{Wnt5}, \textit{Casp6}, or \textit{Foxo4} which encode genes involved in aldehyde dehydrogenase, cancer, apoptosis, or metabolism, respectively were identified in HFD fed mice after GW4064 activation. It was surprising that FXR binds to these genes in the livers of mice fed HFD but not in ND, although FXR binding to the intronic region of gene \textit{Wnt5} was also identified in the ND group.

Subsequently, 21 randomly selected FXR binding regions were subjected to ChIP-qPCR using
pulled ChIP-DNAs from 3 independent ChIP assays. Binding of 20 out of 21 sites (95.2%) was validated showing FXR enrichment on the selected sites after GW4064 activation in HFD fed mice (Fig. 3-7).

The total number of hepatic FXR binding sites (15,263) in the ND group with GW4064 treatment was much more than those (5,272) in HFD fed mice with GW4064 activation which may be partially explained by the finding that expressed endogenous FXR levels are reduced in fatty livers compared to normal livers (Fig. 3-8). Also highly and constitutively acetylated FXR at K217 in obese mice fed HFD support this data since acetylated FXR showed impaired DNA binding (16).

**Location of FXR binding sites**

The hepatic FXR binding sites identified after GW4064 treatment either in ND (15,263) or HFD group (5,272) were then analyzed to identify locations of binding sites. Binding sites with 10 kbp of a gene were assigned to that gene and shown in % value. As shown in Fig.3-9A, FXR primarily bound to intergenic- and intron regions in both groups, composing 78% in ND and 79% in the HFD fed mice. The FXR binding sites within 10 kb upstream of the TSS, within 10 kb of TES, 5’ UTR, or 3’ UTR were indicated as 9.3%, 9.3%, 0.4%, and 0.8%, respectively in ND mice, and 9.2%, 8.4%, 0.2% and 0.7%, respectively in HFD mice (Fig. 3-9A).
However, no noticeable differences in the locations of FXR binding sites were detected in HFD fed mice compared to ND fed mice. Moreover, a similar percentage of hepatic FXR binding sites in the intragenic region was reported in the previous study (13).

Next we examined the FXR binding sites according to the distance from transcription start sites (TSSs), since a higher percentage of regulatory binding sites would be expected near the TSS. Hepatic FXR binding was enriched around ± 1,000 bp from TSS in both diet groups, although distribution of FXR binding around TSS was not different between the two groups (Fig. 3-9B).

**Motif search**

Our group reported that acetylation of FXR is highly elevated in obese mice suggesting acetylated FXR might bind to a different FXRE than unmodified FXR. Thus FXR binding motifs unique to the obese mice were observed using MEME (http://meme.nbcr.net/), a de novo motif search. This assay used the top 250 FXR binding peaks with exception of simple repeats generating 6-25 bp of frequently found bases (21). The analysis of both groups almost exclusively yielded the IR-1 motif as a FXR/RXRα binding motif with a significant statistical value (p-value: 1.895e-06 and 1.173e-05 for ND and HFD, respectively) when compared to known motifs in TOMTOM (Fig. 3-9C). There were no significant differences between the ND
and HFD groups, so no evidence was obtained for different binding motifs in the HFD samples.

Our analysis of FXR binding motif in the mice fed ND was in agreement with the previous report (13).

**Unique FXR bindings identified only in ND or HFD fed mice**

Next we examined whether FXR binding sites in normal mice are same or different from those in HFD induced metabolic disease mice. Two sample comparison (<0.01 FDR) using 15,632 and 5,272 binding sites in ND and HFD mice, respectively generated unique FXR binding sites of 7,440 and 2,344 in ND and HFD mice, respectively (Fig 3-10A). This analysis was of interest, because the unique FXR binding sites in normal liver and fatty liver may be involved in the altered gene regulation by FXR in disease states.

In order to characterize these unique FXR binding sites in each group, the locations of FXR binding and binding frequency around the TSS were examined as described above. This analysis revealed that unique FXR sites were mostly detected in intergenic- and intron region, as 79% in ND and 84% in HFD group. Unique FXR binding in the region of within 10 kb of TES and TSS, exon, 5’UTR, and 3’UTR were shown in Fig. 3-10B. It is noticeable that unique FXR binding to 3’ UTR or 5’ UTR (0.07%) in HFD group is less than those in ND group. FXR binding to exon region in HFD (1.4%) was smaller than that in ND (3.1%) group. However,
distribution frequency of FXR binding sites by distances from the TSS showed similar results in both diet groups, showing highly enriched FXR binding within ± 1,000 bp from the TSS (Fig.3-10C).

Next, examples of unique FXR binding peaks in ND detected at the selected loci using the UCSC genome browser such as Atg2a, Sc5d, Aldh1l2, Pgm2l1, and Gcnt7, genes that are highly involved in liver metabolism are shown in Fig. 3-11. FXR binding peaks were larger after GW4064 treatment in normal liver, but were not increased or not detected in the HFD group. These results such that FXR binding of these sites is required for metabolism or is a consequence of metabolism in the normal liver. Subsequently, the selected unique FXR binding sites in ND were subjected to ChIP-qPCR to validate the ChIP-seq data. When 15 binding sites were selected for ChIP-qPCR using 3 independent ChIP-DNAs, 13 out of 15 (87%) showed significantly increased FXR bindings on to the designated region (Fig. 3-12A).

Likewise, unique FXR binding sites only detected in HFD group were analyzed in a similar way. Genomic location is indicated at the top of the figures and FXR binding peaks from mice treated with vehicle or GW4064 are shown on the UCSC genome browser (Fig. 3-13). In the intronic region of Musk, FXR binding peaks were detected in all the samples but the peaks of FXR with GW4064 treatment in HFD was the largest. FXR binding peaks on Ugdh, Pla1a, and Cma2 were identified only in HFD with GW4064 activation but not in normal liver. When
randomly selected 5 loci were used for ChIP-qPCR using 3 independent ChIP-DNAs, all of them showed FXR recruitment to the binding sites as shown in Fig.3-12B. Ninety percent of the unique FXR binding peaks in either ND or HFD samples detected in the ChIP-seq studies were validated by standard ChIP suggesting FXR binding is functionally significant and might be correlated with biological functions of nearby genes.

**Altered mRNA levels by FXR binding in normal or disease livers**

To answer the question whether FXR binding is functionally correlated with transcriptional regulation of nearby genes, selected genes were subjected to qRT-PCR using total RNAs extracted from the livers of mice treated with GW4064 and fed ND or HFD (n=3/group). First, the mRNA levels of genes occupied by FXR only in ND group were measured and compared to the HFD group. Fifteen out of 30 genes showed either significantly increased or decreased mRNA levels in HFD compared to normal(Fig.3-14A). Since FXR binding was not detected in HFD, this result suggests that unique FXR binding in normal livers correlates with altered gene regulation. To infer whether these FXR binding genes respond to GW4064 signaling in normal livers, gene expression of the selected genes (*Atg2a, Ptpfr, Oxct2b, Pgm2l1, and Sc5d*) was examined in qRT-PCR. We used normal livers with or without GW4064, because we do not know whether bile acid signaling is normal in HFD. All the selected genes had induced mRNA
levels after GW4064 treatment in normal livers showing these genes are responsive to GW4064 as shown in Fig. 3-15. This data strongly suggests that gene regulation by FXR activation is impaired in the metabolic disease group due to the absence of FXR binding.

It is interesting that lack of FXR binding sites in HFD at loci compared to ND correlated with increased expression of the genes in HFD compared to ND. This would suggest that FXR binding represses expression of these genes even though FXR is generally considered a transcriptional activator. These genes included genes involved in energy metabolism, Sc5d (Sterol C5-desaturase) encoding lathosterol oxidase which is an enzyme of cholesterol biosynthesis and Gcnt7 (N-acetylglucosaminyl transferase) which belongs to the family of glycosyltransferases. Genes encoding a solute carrier (Slc7a15), mannosidase (Man1b1), and guanylate cyclase (Guca1a) which catalyze GTP to cGMP were also represented. In contrast, signaling genes encoding phosphatases (Ppp2r5c and Ptprf) were found to have significantly reduced mRNA levels in HFD compared than in ND fed mice. This data represents that these genes were not induced by FXR in HFD group which implies that metabolic regulation by FXR was impaired in the HFD fed group.

Interestingly, a gene encoding TNF alpha superfamily 4 (Tnfsf4), an important inflammation stimulus was significantly elevated in disease livers compared in healthy livers. This indicates that FXR may partially be involved in inflammation of fatty livers that is
consistent with the fact that chronic inflammation is associated with fatty livers (23).

Furthermore, genes encoding proteins in autophagy, cell cycle or cancer like Atg2a (autophagy related 2 homolog), Cdca4 (cell division cycle associated 4), and Mas1 (Mas oncogene) that are regulated by FXR were not regulated in disease which is supporting previous findings (24, 25).

These data strongly suggest that FXR binding sites detected only in normal livers are highly involved in lipid metabolism, inflammation, cell cycle and tumorigenesis, implying that these functions might not be abnormally regulated displaying altered gene expressions in the disease liver.

Similarly, 28 genes having unique FXR binding sites in HFD were subjected to qRT-PCR to examine relative mRNA levels in normal and disease livers as performed previously with genes having unique FXR bindings in HFD sample. In our analysis, only 5 genes like Musk, Ugdh, Hic2, Cma and Pla1a showed significantly altered mRNA levels in disease livers when compared to normal livers (Fig. 3-14B). The mRNA levels of these genes were both upregulated (1/5) and downregulated (4/5) suggesting that FXR binding may be associated with either activation of repression. Pla1a (phospholipase A1 member) and Ugdh (UDP-glucose dehydrogenase) are involved in lipid and energy metabolism, while Hic2 (hypermethylated in cancer 2), Cma2 (chymase 2), and Musk (Muscle, skeletal, receptor tyrosine kinase) are in cancer and signaling pathway suggesting these aberrant regulation of these genes by FXR may play
important roles for liver cancer and pro-inflammation reaction as well as metabolism in liver.

However, it should be noted that 23 out of 28 genes having unique FXR binding sites in HFD group did not show significant changes in mRNA levels from ND mice. This might be due to functionally inactive FXR in HFD which is unable to regulate its mRNA levels since FXR is highly and constitutively acetylated in the HFD fed mice, subsequently its interaction with RXRα and DNA binding was impaired. Or those FXR bindings might be from the experimental variation or false positive data.

**Functional gene ontology (GO) of unique FXR binding**

To reveal the functional biological pathways of unique FXR bindings in either ND or HFD group which may be involved in the disease pathways, gene ontology (GO) was performed with a maximum statistical stringency (27). This analysis for 1,566 or 2,583 of genes having unique FXR bindings in HFD or ND, respectively revealed several functional categories for the closest genes identified in DAVID (Database for Annotation, Visualization, and Integrated Discovery) (Fig. 3-10A) (20). The enrichment score in Table 3-4 establishes the level of stringency while clustering the genes in GO analysis. Due to the hierarchial structure of GO categories, the same gene can be assigned with multiple GO terms (28). Unique FXR binding pathways included groups of genes encoding Ca\(^{2+}/K^+\) channels, S/T kinases, and lipid oxidation
enzymes in normal liver, while iron-sulfur binding proteins, Toll like receptor and oncogenic proteins were in fatty livers (Table 3-4). This analysis is of interest, because functional pathways detected using unique FXR binding sites in normal livers indicate genes that are regulated in normal, but not in diseased livers due to the absence of FXR binding. Conversely, genes detected with unique FXR binding sites in HFD group indicate genes aberrantly regulated by FXR binding in disease liver.
**Discussion**

To unravel the target gene network of a transcription factor in pathophysiology, identification of its interaction sites, *cis*-regulatory elements, is essential (11). This current study not only compares whole genome FXR binding sites in normal liver and fatty livers induced by high fat diet, but also identifies unique FXR binding sites in HFD mice that potentially underlie abnormal gene regulations. Likewise, unique FXR binding detected only in fatty livers might be highly involved in the disease, because these bindings were not detected at all in normal livers. Further, unique FXR binding sites detected only in normal livers, which may indicate the loss of regulation of critical genes for the metabolic disease. Although unique FXR binding and subsequent transactivation should be examined thoroughly, our investigations indicate different hepatic FXR binding to distinct classes of genes in the normal and disease states.

The initial validation of ChIP assays by PCR or qPCR showed that endogenous FXR was already bound to the regulatory regions of FXR targets even in the untreated mice because fed mice have same levels of endogenous FXR ligands. Similarly, in the ChIP-seq study, the UCSC genome browser displayed small FXR binding peaks on vehicle and GW4064 treated samples at the same locations (Fig. 3-4). The binding peak sizes, however, was markedly enhanced upon treatment with GW4064, which has a higher affinity to FXR than the endogenous ligands as reported (13).
Our genome-wide maps of FXR occupancy identified with high confidence 15,263 FXR binding sites with GW4064 activation in normal livers, and 5,272 in fatty livers. Differences in the total number of sites can be partially explained by the fact that FXR levels in fatty liver are reduced compared to normal liver when whole cell extracts from mouse livers were used for western blotting (Fig. 3-8), which is consistent with a previous report (4). In addition, our group reported that FXR is highly and constitutively acetylated at K217 in FXR in the livers of genetic obese mice (ob/ob) and diet induced obese mice (16). As a result of acetylation, FXR impairs DNA binding and heterodimerization with RXRα in vivo and in vitro. These previous reports are consistent with our data in which the number of FXR binding sites in disease livers is less than in normal livers.

Hepatic FXR binding analyses showed that the FXR binding location relative to genes, the frequency of FXR binding distribution around the TSS, and binding motifs are similar in both diet groups, although number of binding sites is different (Fig. 3-10). It was surprising that FXR which assumed to be post-translational modified, exhibits elevated acetylation in fatty liver has similar characteristics for binding location, binding frequency around TSS, and motifs. However, unique FXR binding sites in diseased liver was identified indicating these FXR binding sites are novel compared to normal liver and possibly related with genes underlying pathogenesis. Therefore, unique FXR binding DNA regions (2,344) in fatty livers throughout the entire mouse
genome were analyzed for functional annotation in GO and were compared with those (7,440) in normal livers. It demonstrated that FXR is functionally involved in different biological pathways in diseased liver compared to normal (Table. 3-4). Clusters containing genes occupied by FXR in disease were highly enriched for toll-like receptor signaling pathways as well as peroxisome, reflecting the changes of inflammatory genes that are associated with the metabolic diseases in liver (26). Also, FXR binding in the promoter region of Tnfsf4, which is a proinflammatory cytokine initiating innate immune responses was detected only in normal livers (Fig. 3-4).

Furthermore, it has been reported that FXR binding in iNOS promoter regions was markedly increased when cells were treated with LPS (23), and the change in binding correlated with sumoylation of FXR, a post-translational modification (23). Our data support the idea that FXR is highly involved in the regulation of inflammatory reactions and may play a role in chronic low-grade inflammation that plays a key role in the initiation, propagation, and development of metabolic diseases as reported (26).

In the GO analysis, the most significantly clustered group of genes in HFD group were iron-sulfur binding proteins which are best known for their roles in oxidation-reduction of mitochondrial electron transport (29), which suggests FXR binding in disease may be involved in energy metabolism, although further studies are required. Another cluster contains genes like apoptosis and Ras oncogenic proteins in HFD group is in agreement with the finding that FXR
plays an important role for hepatocarcinogenesis (30). It also supports previous reports indicating
important functions of FXR in tumorigenesis in liver (31). GO analysis selected gene for which
the FXR binding sites was within ± 10 kb of upstream or downstream TSS for annotation, so it
is more likely that some potential target genes which have FXR binding sites > 10kb from the
gene were not identified.

To determine whether there is a correlation between gene expression and FXR binding, 28 randomly selected genes showing FXR binding only in HFD group were subjected to qRT-PCR to measure gene expressions. Levels of mRNA were significantly altered in only 5 out of 28 genes compared to ND as described in Result. This result indicates that functional activity of FXR as a transcriptional regulator is possibly impaired in metabolic diseases even though its binding was still detected. If the 28 genes are a representative sample, about 80% occupied by FXR in fatty livers would not be regulated by activation of FXR.

On the other hand, 50% of genes (15 out of 30 genes) occupied by FXR only in normal livers showed significantly altered gene expressions compared to disease livers. This result suggests that hepatic FXR should regulate its target properly by binding upon bile acid signaling, however FXR occupancy on to its cistrome was not detected in the disease, which may be responsible for gene regulations involved in the metabolic disease.
One of the interesting observations in this study is that gene regulation by FXR binding are negative as well as positive in ND compared to HFD for energy metabolism, inflammation, cell signaling and a cell cycle. Positively regulated genes were involved in energy metabolism, inflammation, and cancer, while genes for kinases, autophagy, cell cycle, and energy metabolism are negatively regulated by FXR binding in normal liver (Fig. 3-14A). This is in agreement with previous report that FXR negatively regulates ApoAI expression in human liver cells by binding to its negative element (8). Also, the nuclear receptor PXR (Pregnane X receptor), which is known as a key regulator of xenobiotic metabolism in liver, has recently been revealed by ChIP-seq analysis to have both induction and suppression functions in gene regulation (32).

It is noteworthy that Zhang et al. published data showing hepatic FXR activation by GW4064 for 4 or 11 days in db/db mice changed gene expression of G6pase and Pepck, which were responsible for hyperglycemia and hyperlipidemia in liver (4). This supports the idea that FXR function is impaired for gene regulation in diseased mice, but activation of FXR for a long term finally regulates its target correctly generating beneficial outcomes. Another report by Jiang et al. showed that renal FXR activation using GW4064 for 1 week in db/db mice changed mRNA expressions of Srebp-1c and Scdl for fatty acid synthesis, and Mcp-1 and Tnf α for proinflammatory cytokines (33). Thus, to observe whether FXR mediated-gene expression is modified after long term treatment of GW4064 for FXR activation, obese mice were treated by
GW4064 for 5 days. However, none of the genes that were regulated by FXR binding were significantly altered by GW4064 treatment in disease mice (Fig. 3-15). It is not clear why these genes are not regulated by FXR activation in disease. Also FXR may be silent factor in diseased mice so result may not correlated directly with FXR binding. In fact, due to the limitation of ChIP-seq assay, indirect FXR binding on to genomes can not be ruled out. Thus, it is not known whether all the FXR bindings are through direct binding or tethering to the target DNA. Therefore, further studies are required to understand how FXR directly regulates genes by binding in the obese mice or significance of FXR’s involvement in the disease. Molecular mechanisms by which FXR regulates differentially its target in disease liver should be studied thoroughly.

In conclusion, these data provide a comprehensive genome-wide map of FXR occupancy in normal and disease liver, at least to our knowledge, the first approach to directly compare global FXR binding profiles in normal and diseased livers. However, to unravel a correlation between the genome wide screen of FXR bindings and expressions of corresponding genes in disease, a microarray analysis using livers of obese mice induced by high fat diet will be required. For the selected genes occupied by FXR analyzed in this study altered gene expressions was associated with FXR binding. These findings are intriguing in terms of development of
therapeutic agents targeting FXR to cure metabolic diseases including type II diabetes, cardiovascular disease, and obesity.
Table 3-1

Table 3-1. Summary of animal profiles used for ChIP-seq analysis.

Body weight of mice fed HFD was significantly increased than in ND group. ** indicates p<0.001 by student’s t-test.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Group of mice</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>ND</td>
<td>HFD</td>
<td></td>
</tr>
<tr>
<td>Number of mice</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Body weight (g, mean ± SEM)</td>
<td>29.02 ± 1.28</td>
<td>31.35 ± 0.63</td>
<td>35.75 ± 0.53**</td>
</tr>
<tr>
<td>Ages (month, mean ± SD)</td>
<td>7.7 ± 2.6</td>
<td>8.5 ± 1.3</td>
<td>9.5 ± 0.7</td>
</tr>
<tr>
<td>GW4064 (30mg/Kg/day)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3-2

Table 3-2. Mapping rates of FXR ChIP-DNAs for identification of hepatic FXR binding sites in all samples. The sequenced tags for FXR ChIP-seq assays are listed in each animal group with vehicle or FXR agonist, GW4064 treatment. The number of peaks was identified with a FDR of < 0.01 in CisGenome.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Ab</th>
<th>FXR Ligand</th>
<th>Total reads</th>
<th>Mapping rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Diet</td>
<td>FXR</td>
<td>Vehicle</td>
<td>5,012,745</td>
<td>54.2</td>
</tr>
<tr>
<td></td>
<td>FXR</td>
<td>GW4064</td>
<td>2,985,933</td>
<td>49.8</td>
</tr>
<tr>
<td>High Fat Diet</td>
<td>FXR</td>
<td>Vehicle</td>
<td>6,051,048</td>
<td>64.7</td>
</tr>
<tr>
<td></td>
<td>FXR</td>
<td>GW4064</td>
<td>3,972,513</td>
<td>55.5</td>
</tr>
</tbody>
</table>
Table 3-3

Table 3-3. Identification of FXR binding sites in each sample.
Threshold of each sample was indicated with <0.01 FDR.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Groups</th>
<th>Threshold (Min.size of peak)</th>
<th>Num. of peaks</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>FXR + vehicle</td>
<td>15</td>
<td>39,234</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>FXR + GW4064</td>
<td>13</td>
<td>71,434</td>
<td>0.01</td>
</tr>
<tr>
<td>HFD</td>
<td>FXR + vehicle</td>
<td>9</td>
<td>75,862</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>FXR + GW4064</td>
<td>12</td>
<td>22,931</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Table 3-4

Table 3-4. Gene Ontology (GO) analysis of unique FXR binding sites in the ND or HFD fed mice group using DAVID web tool (http://david.abcc.ncifcrf.gov).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Functional Annotation</th>
<th>Enrichment score</th>
<th>P-value</th>
<th>Num.of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>SH2 domain</td>
<td>2.46</td>
<td>1.70E-03</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Ca2+,K+ channel</td>
<td>2.06</td>
<td>6.88E-03</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Sodium transport</td>
<td>1.98</td>
<td>1.39E-02</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Metal ion binding</td>
<td>1.95</td>
<td>8.21E-03</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>G-protein signaling</td>
<td>1.74</td>
<td>1.18E-02</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>cAMP regulation</td>
<td>1.41</td>
<td>2.87E-02</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>S/T kinases</td>
<td>1.30</td>
<td>3.57E-02</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Nuclear receptor</td>
<td>1.13</td>
<td>2.28E-02</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Lipid (FA) oxidation</td>
<td>1.08</td>
<td>4.93E-02</td>
<td>4</td>
</tr>
<tr>
<td>HFD</td>
<td>Iron-sulfur binding</td>
<td>2.59</td>
<td>2.03E-03</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Metal Ion binding</td>
<td>1.95</td>
<td>7.72E-03</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>Apoptosis</td>
<td>1.77</td>
<td>1.50E-02</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Ras Oncogenic proteins</td>
<td>1.70</td>
<td>1.48E-02</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Peroxisome</td>
<td>1.60</td>
<td>1.94E-02</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Neurotransmitter degradation</td>
<td>1.48</td>
<td>1.53E-02</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Toll-like Receptor signaling pathway</td>
<td>1.03</td>
<td>1.53E-02</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 3-5

Table 3-5. List of primers for qRT-PCR of mouse genes having unique FXR binding sites.

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Musk</td>
<td>cccagatgtgtcgtctttgga</td>
<td>acagagacagtggtggaggaac</td>
</tr>
<tr>
<td>2</td>
<td>Cma2</td>
<td>gaaccacactcccgaccttaa</td>
<td>tggcagcgcatcatacaca</td>
</tr>
<tr>
<td>3</td>
<td>Hic2</td>
<td>ctccccatgttgctcagcaga</td>
<td>aactcagggcagctggagga</td>
</tr>
<tr>
<td>4</td>
<td>Pla1a</td>
<td>cctaaaccacagtggcacat</td>
<td>atgcagaccagctcctctgct</td>
</tr>
<tr>
<td>5</td>
<td>Ugdh</td>
<td>cgcataatgtgacccaacac</td>
<td>tccggtcctttagctcct</td>
</tr>
<tr>
<td>6</td>
<td>Atg2a</td>
<td>cgctcggaacttgctcgtc</td>
<td>tcttgacctcagtgctcct</td>
</tr>
<tr>
<td>7</td>
<td>Ppp2r5c</td>
<td>cccagaagagagatgaacca</td>
<td>tggggtggaatatctggagac</td>
</tr>
<tr>
<td>8</td>
<td>Ptprf</td>
<td>acccagatgtgtcagttaca</td>
<td>gcctcccctgttttggtta</td>
</tr>
<tr>
<td>9</td>
<td>Cdc4</td>
<td>aagggaggaatgagccagag</td>
<td>tcccctcactgtacgacaca</td>
</tr>
<tr>
<td>10</td>
<td>Oxct2b</td>
<td>cttggccgaccaactcactca</td>
<td>cgctgcacactcctcattt</td>
</tr>
<tr>
<td>11</td>
<td>Stl7a15</td>
<td>tctcccccaacatacagag</td>
<td>tgcacaggaacaggtataag</td>
</tr>
<tr>
<td>12</td>
<td>Aldh1l2</td>
<td>gaggtgaacgaggtgacagc</td>
<td>cactcttgtgggtggcagtg</td>
</tr>
<tr>
<td>13</td>
<td>Pgm2l1</td>
<td>agcgaccagcccaataagaa</td>
<td>aatcctgaagcagccgagt</td>
</tr>
<tr>
<td>14</td>
<td>Man1b1</td>
<td>tgtgaacattggcactggat</td>
<td>ctccacatgatgtcatcga</td>
</tr>
<tr>
<td>15</td>
<td>Gcnt7</td>
<td>tgtgcctctcatctctttt</td>
<td>aagccgagcacaggtcatct</td>
</tr>
<tr>
<td>16</td>
<td>Guca1a</td>
<td>gtgcgaggagataccagat</td>
<td>atctgtgtccctctgcaccc</td>
</tr>
<tr>
<td>17</td>
<td>Sc5d</td>
<td>ctctacacasagacacccc</td>
<td>gctctcactctctcccaca</td>
</tr>
<tr>
<td>18</td>
<td>Tnfsf4</td>
<td>cccttggtgagatactggaa</td>
<td>cagagacacacagcttaagc</td>
</tr>
<tr>
<td>19</td>
<td>Esrrb</td>
<td>aacctggtggaccagatgag</td>
<td>ctaccagggcagaggtgtcc</td>
</tr>
<tr>
<td>20</td>
<td>Mas1</td>
<td>ttggtgaccaccatggagta</td>
<td>gcgaggtggaagacacaagag</td>
</tr>
<tr>
<td>21</td>
<td>Nr0b2</td>
<td>cagtgagaacccctgtctt</td>
<td>ctggcccaaaacaccggac</td>
</tr>
<tr>
<td>22</td>
<td>Ostb</td>
<td>ccgcaatgagacgatcatac</td>
<td>gtaatgaccacccagaaatg</td>
</tr>
</tbody>
</table>
Table. 3-6

Table 3-6. List of primers for ChIP-qPCR of ChIP-seq analysis

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ostα</td>
<td>ATGCACGATGTGTCTGTGTGT</td>
<td>GCACACGCATTTCATAGAC</td>
</tr>
<tr>
<td>2</td>
<td>Ostβ</td>
<td>ATGCATGCTCCCACTGAGCA</td>
<td>GCAGATCATTACTGGGCTCT</td>
</tr>
<tr>
<td>3</td>
<td>Pltp</td>
<td>GTCTCCGAGCTGCTGCTGTC</td>
<td>TGTCGTTGGGTAAGGTAGC</td>
</tr>
<tr>
<td>4</td>
<td>Tm9sf1</td>
<td>GCACGGGATACGGGTAGGAGCA</td>
<td>AGGGTAAATGGGACTCTCT</td>
</tr>
<tr>
<td>5</td>
<td>Pcloa</td>
<td>GAAGGATGGAGCTGAGCTGCA</td>
<td>GCGACAGGGACTGGCAGAT</td>
</tr>
<tr>
<td>6</td>
<td>Cyp8b1</td>
<td>TTGGGTCCACGGCCTCGAG</td>
<td>AGGTGTTGTAAGGTGACTCTCT</td>
</tr>
<tr>
<td>7</td>
<td>Atg3</td>
<td>CGAGGTTCGATGCCAGCTG</td>
<td>CAGAGGGACTGGCATCAC</td>
</tr>
<tr>
<td>8</td>
<td>Nr1i3</td>
<td>TGATATGAGGTGTGGTGGAGCAA</td>
<td>GTGGGTAAATGGGACTCTCT</td>
</tr>
<tr>
<td>9</td>
<td>Man2c1</td>
<td>GAACAAAGTTCCCGCCACT</td>
<td>AAAGCAGTCTCTCTGACTTA</td>
</tr>
<tr>
<td>10</td>
<td>Ppp2cb</td>
<td>TGTGCACTCACTGAGCTGCA</td>
<td>CAGACACCCCACTACAAA</td>
</tr>
<tr>
<td>11</td>
<td>Hlf</td>
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<td>CAGACACCCCACTACAAA</td>
</tr>
<tr>
<td>12</td>
<td>Gcnt7</td>
<td>AAGGCCATTCCTCTCCCTCAT</td>
<td>CAGACACCCCACTACAAA</td>
</tr>
<tr>
<td>13</td>
<td>Tnfsf4</td>
<td>AAATTTTGTTGACATGTTGTG</td>
<td>CAGACACCCCACTACAAA</td>
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<tr>
<td>14</td>
<td>Art2b</td>
<td>GACAGGTAGGCTGCTGAGGAA</td>
<td>ACATGACACCATGAC</td>
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<tr>
<td>15</td>
<td>Man1a</td>
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<td>AGGGTAAATGGGACTCTCT</td>
</tr>
<tr>
<td>16</td>
<td>Plau</td>
<td>GTTCAGATGGACTGACAGGTA</td>
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<tr>
<td>17</td>
<td>Pcdhgc4</td>
<td>CAATCCACATACCACACAGA</td>
<td>CAGACACTCCACATACATGCT</td>
</tr>
<tr>
<td>18</td>
<td>Pcloa</td>
<td>CCCTGCACTGAGCTCAGACTGGAG</td>
<td>CAGACACTCCACATACATGCT</td>
</tr>
<tr>
<td>19</td>
<td>Hic2</td>
<td>TTAGAAGGGGTGGGCAAGA</td>
<td>CAGACACTCCACATACATGCT</td>
</tr>
<tr>
<td>20</td>
<td>Hlf1a</td>
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<td>Mettl1</td>
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<tr>
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<td>TCAGTACCAAGCAGGCAAA</td>
</tr>
<tr>
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<td>Cma2</td>
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<td>GACATGCTAGGGCATCATGCT</td>
</tr>
<tr>
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<td>Wnt5a</td>
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<tr>
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<tr>
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<tr>
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</table>
Table 3-6 (Cont.)

Table 3-6. List of primers for ChIP-qPCR of ChIP-seq analysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
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<td>ACGGCTTCATGCACACTAGG</td>
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<tr>
<td>33</td>
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Fig. 3-1

(A) Levels of mouse Srebp-1c and Scd1 mRNA in the HFD fed mice determined by qRT-PCR (n=4/group). Asterisk (*) indicates p-value of <0.05 by Student’s t-test.

(B) Relative mRNA levels of mouse Shp determined by qRT-PCR in the livers of ND or HFD after 1 h of GW4064 activation.

Fig. 3-1. Relative mRNA levels of target genes in the livers of mice fed ND or HFD.

(A) Levels of mouse Srebp-1c and Scd1 mRNA in the HFD fed mice determined by qRT-PCR (n=4/group). Asterisk (*) indicates p-value of <0.05 by Student’s t-test.

(B) Relative mRNA levels of mouse Shp determined by qRT-PCR in the livers of ND or HFD after 1 h of GW4064 activation.
Fig. 3-2

(A) ChIP assay

GW4064 - + - +

Total

-Ag

FXR

Pol II

(B) FXR ChIP

Fold enrich

GW4064 - + + +

High Fat Diet - + + +

RXR ChIP

Fold enrich

(C) FXR ChIP

Fold enrich

GW4064 - + + +

High Fat Diet - + + +

RXR ChIP

Fold enrich

Fig. 3-2. ChIP analysis for ChIP-seq. (A) ChIP-conventional PCR represents FXR recruitment by GW4064 treatment on the mouse Shp promoter region or Gapdh promoter as a control in normal mice. ChIP-qPCR represents FXR recruitment on Shp (B) and Ostb (C) promoter region in all the animal groups. RXR recruitment on the same region was showed as a control.
Fig. 3-3. Venn Diagram demonstrates hepatic FXR binding sites after GW4064 treatment for FXR activation in ND and HFD fed groups. High confident FXR binding sites were generated with statistical cutoffs including a FDR of <0.01 and ratio of IgG peaks to FXR (<5).
Fig. 3-4. Examples of FXR binding peaks on the selected loci in the livers of ND fed mice after GW4064 treatment.

FXR recruitment on the promoter regions of Nr0b2(Shp), Ostb, Cyp8b1, and Tnfsf4 were visualized on the UCSC mouse genome browser. Genomic locations were indicated at the top of the figure.
Fig. 3-5

Randomly selected 15 binding sites were adapted to ChIP-qPCR to validate recruitment of FXR on to the selected loci using pulled ChIP-DNAs from three independently performed ChIP assays.

FXR recruitment on Nr0b2 (Shp) was repeated more than 3 times using independent ChIP DNAs. Mean ± SEM was indicated in Nr0b2.
Fig. 3-6. FXR binding peaks on the randomly selected loci including (A) Adh7, (B) Wnt5, (C) Casp6, and (D) Foxo4 on the UCSC genome browser. Mice fed HFD were treated with vehicle or GW4064 to activate FXR. Genomic locations are indicated at the top of the figures.
Fig. 3-7

Randomly selected 21 FXR binding sites were used for ChIP-qPCR to validate FXR binding on to the selected loci in the HFD group.

ChIP-qPCR used pulled ChIP-DNAs obtained from three independent ChIP assays.
Fig. 3-8. Expressed levels of endogenous hepatic FXR of mice fed ND or HFD.
(A) Expressed mRNA levels of hepatic FXR by qRT-PCR (n=4/group). (B) Expressed protein levels of endogenous FXR in whole liver extracts was detected by immunoblotting after immunoprecipitation using FXR antibody.
Fig. 3-9

A

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<th>Locations</th>
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<tr>
<td>3' UTR</td>
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B

C

Fig. 3-9. Analyses of FXR binding sites in the livers of mice fed ND or HFD with GW4064 activation.

(A) Location of FXR binding sites in ND or HFD fed mice with GW4064 activation relative to known genes. TSS; within 10 kb of Transcriptional start site, TES; within 10 kb of Transcriptional end site, UTR; Untranslated region of 3’-and 5’-end. (B) Distributions of FXR binding peaks (15,263 and 5,272) around TSS (upstream and downstream ± 4,000 bp) was shown in plot. (C) Motif analysis of hepatic FXR binding sites using top 250 peaks in ND or HFD groups with GW4064 activation in MEME.
Fig. 3-10. Analyses of unique FXR binding sites detected only in the livers of mice fed ND or HFD.

(A) Venn Diagram demonstrating the unique FXR binding sites (<0.01 FDR) in either ND or HFD group. The number of genes with nearby FXR binding sites are indicated in parenthesis. (B) Location of unique FXR binding peaks relative to known genes was shown in % value using 7,440 and 2,344 of FXR binding sites. (C) Distributions of FXR binding peaks (7,440 and 2,344) around TSS (upstream and downstream ±4,000 bp) was shown in plot.
Fig. 3-11. Unique FXR binding peaks detected in normal livers with GW4064 activation were presented on the UCSC genome browser.

FXR binding peaks with Vehicle or GW4064 were shown in both diet groups on the selected loci including (A) Atg2a, (B) Sc5d, (C) Aldh1l2, (D) Pgm2l1, and (E) Gcnt7. Genomic location is indicated at the top of the figure.
Fig. 3-12. FXR recruitment on the selected loci by FXR ChIP-qPCR. The data represents qPCR using pulled ChIP-DNAs from 3 independent ChIP assays. For the statistical significance, 2 independent ChIP-qPCRs were performed and shown as the average ± SEM with statistical significance by Student t-test (* p<0.05, ** p<0.01, ***p<0.001). FXR ChIP-qPCR was normalized to a non-target genomic site and error bar represents mean ± SEM from independent biological replicates. (A-B) Fold changes of FXR recruitment in ND and HFD group.
Fig. 3-13. Unique FXR binding sites detected only in HFD group with GW4064 activation were displayed on the selected loci (A) *Musk*, (B) *Ugdh*, (C) *Pla1a*, and (D) *Cma2* using the UCSC genome browser.
Fig. 3-14

**Fig. 3-14. Relative mRNA levels of genes containing unique FXR binding sites in ND (A) or HFD (B).**

Total RNA extracted from the livers of mice fed ND or HFD with GW4064 activation overnight was used for RT reaction and qPCR was performed (n=3/group). Mean values are shown ± SEM. Abbreviations: Infm; Inflammation, NR; Nuclear receptor.
Fig. 3-15. Relative mRNA levels of genes (Atg2a, Ptprf, Oxct2b, Pgm2l1, and Sc5d) containing unique FXR binding sites in normal livers after GW4064 treatment.
References


Genome-wide analysis of SREBP-1 binding in mouse liver chromatin reveals a preference for promoter proximal binding to a new motif. Proc Natl Acad Sci U S A. 106: 13765-13769


Curriculum Vitae

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Education

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Ph.D School of Molecular and Cellular Biology
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2002-2004 Molecular biology
Master of Science Graduate School of Life Sciences and Biotechnology
Korea University, Seoul, Korea

1995-2000 Animal Science
Bachelor of Agriculture Korea University, Seoul, Korea

Fellowship and Awards

- Excellent Teaching Assistant (UIUC): Fall 2008
- Graduate Fellowship in National Research Foundation of Korea:2005-2007
- Excellence in Academic Achievement
  (Korea Institute of Science and Technology): Feb. 2004
- Graduate fellowship in Molecular and Cellular Biology (UIUC); Fall 2005
- Honors scholarships (Korea Univ): Fall 1998, Spring 1999
- First Class Honors (Korea Univ): Spring 1998, Fall 1998, Spring 1999

Activities

- Committee of MIP Student Seminar (UIUC): Fall 2009-2011
- Member of Molecular Endocrinology society: 2009-present
Teaching Experiences

- MCB 101: Introductory Microbiology Lab, MCB, UIUC
- MCB 151: Molecular and Cellular Basis of Life Laboratory, MCB, UIUC
- MCB 251: Experimental Techniques in Molecular Biology, MCB, UIUC
- MCB 301: Experimental Microbiology, MCB, UIUC

Research Experiences

- Research trainee
  Bioelectrochemistry lab. Water Environment & Remediation Research Center
  Korea Institute of Science and Technology (KIST): 2001-2002
- Graduate Research Fellow
  Bioelectrochemistry lab. Water Environment & Remediation Research Center
  Korea Institute of Science and Technology (KIST): 2002-2004

Technical Skills

- Molecular Biology: DNA, RNA, and protein preparation and analysis, RT (reverse transcriptase) reaction, qPCR, RT and qPCR of microRNA, Antisense- miRNA transfection, CoIP, ChIP, Western blotting, Construction of Adenovirus, Amplification and purification of adenovirus
- Cell Biology: Human and mouse cell line culture, Transfection, Confocal microscopy, siRNA knockdown, Tissue histochemistry (Oil red O)
- Biochemical skills: Protein purification, Affinity purification of His-tagged proteins, protein assay, Gel shift assay
- Biotechnology: Gene cloning, PCR, Ligation, DNA gel electrophoresis, Site-directed mutagenesis, RFLP (Restricted fragment length polymorphism), Gene library construction, Bacterial isolation and identification using 16S rDNA
- Animal Experiment: Breeding, Tissue collection, Intravenous and Intraperitoneal injection of drugs and viruses, Oral glucose tolerance test
- Bioinformatics: Gene alignment, ChIP-seq analysis (CisGenome, UCSC browser)
Publications


Presentations


4. **J. Lee**, J.K.Kemper. 2009. FXR positively regulates SIRT1 through miR-34a. MIP Retreat, Department of Molecular & Integrative Physiology, School of Molecular and Cellular Biology, UIUC, IL.

5. **J. Lee** and A.A.Salyers. 2007. Characterization of Orf2c and Orf2d in *Bacteroides* Conjugative transposon, CTnDOT. Allerton conference, Department of Microbiology, School of Molecular and Cellular Biology, UIUC, IL.

6. **J. Lee** and A.A.Salyers. 2006. Purification of His-tagged Orf2c and Orf2d in Bacteroides Conjugative transposon, CTnDOT. Allerton conference, Department of Microbiology, School of Molecular and Cellular Biology, UIUC, IL.


9th International Symposium on Genetics of Industrial Microorganism, 
15. P.T.Nguyet, H.S.Park, J.Y.Lee and B.H.Kim, 2002. Diversity of Microbial population in a Mediator-less Microbial fuel cell, 
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9th International Symposium on Genetics of Industrial Microorganism, 
9th International Symposium on Genetics of Industrial Microorganism, 
18. P.T.Nguyet, H.S.Park, J.Y.Lee and B.H.Kim, 2002. Diversity of Microbial population in a Mediator-less Microbial fuel cell, 
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