Effects of Overexpression of Fibroblast Growth Factor 15/19 on Hepatic Drug Metabolizing Enzymes

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Running Title

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Abbreviations

Ad/EYFP-hCAR	Adenovirus expressing enhanced yellow fluorescent protein-tagged hCAR
BA	Bile acid
CAR	Constitutive androstane receptor
CAR ^{-/-}	CAR knockout mice
DMET	Drug metabolizing enzymes and transporters
ERK	Extracellular signal regulated kinase
FGF15	Fibroblast growth factor 15
<i>Fgf15</i> Tg	<i>Fgf15</i> transgenic mice
<i>Fgf15</i> ^{int-/-}	Intestine-specific <i>Fgf15</i> knockout mice
FGF19	Fibroblast growth factor 19
FGFR4	Fibroblast growth factor receptor 4
FXR	Farnesoid X receptor
GH	Growth hormone
HU	Hounsfield unit
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinases

NAFLD	Non-alcoholic fatty liver diseases
NASH	Nonalcoholic steatohepatitis
NR	Nuclear receptor
PB	Phenobarbital
PHH	Primary human hepatocytes
RT-PCR	Real-time quantitative polymerase chain reaction
WT	Wild type

Abstract

Fibroblast growth factor 15/19 (FGF15/19) are endocrine growth factors that play an important role in maintaining bile acid homeostasis. FGF15/19 based therapies are currently being tested in clinical trials for the treatment of non-alcoholic steatohepatitis and cholestatic liver diseases. To determine the physiological impact of long-term elevations of FGF15/19, a transgenic mouse model with overexpression of *Fgf15* (*Fgf15* Tg) was used in the current study. The RNA-seq analysis revealed elevations of the expression of several genes encoding phase I drug metabolizing enzymes (DMEs), including *Cyp2b10* and *Cyp3a11*, in *Fgf15* Tg mice. We found that the induction of several *Cyp2b* isoforms resulted in increased function of CYP2B in microsomal metabolism and pharmacokinetics studies. Because CYP2B family is known to be induced by constitutive androstane receptor (CAR), to determine the role of CAR in the observed inductions, we crossed *Fgf15* Tg mice with CAR knockout mice and found that CAR played a minor role in the observed alterations in DME expression. Interestingly, we found that the overexpression of *Fgf15* in male mice resulted in a phenotypical switch from the male hepatic expression pattern of DMEs to that of female mice. Differences in secretion of growth hormone (GH) between male and female mice are known to drive sexually dimorphic expression patterns of hepatic genes in a STAT5b dependent manner. We found that male *Fgf15* Tg mice presented with many features similar to GH deficiency, including lowered body length and weight, *Igf-1* and *Igfals* expression, and STAT5 signaling.

Significance: The overexpression of *Fgf15* in mice causes an alteration in DMEs at the mRNA, protein, and functional levels, which is not entirely due to CAR activation but associated with lower GH signaling.

Introduction

Drug metabolism is the process by which exogenous compounds undergo biotransformation in order to facilitate their removal from the body. The process of drug metabolism is described in three phases: functional conversion, conjugation, and transport/excretion. The transcriptional regulation of genes encoding hepatic drug metabolizing enzymes and transporters (DMETs) is a critical mechanism capable of responding to various challenges during development, exposure to xenobiotics, and alterations in physiology and pathology.

The liver is the primary site of drug metabolism. The efficiency or rate at which the liver is able to process xenobiotic biotransformation is dependent upon the relative abundance of DMETs. In order to regulate the expression of DMETs, the liver is capable of responding to xenobiotic exposure through the activation of xenobiotic sensing nuclear receptors (NRs), such as the constitutive androstane receptor (CAR, NR1I3), pregnane X receptor, glucocorticoid receptor, and vitamin D receptor.

CAR is a well-studied xenobiotic sensing NR in the regulation of DMET gene expression. Under normal conditions, CAR is bound to the cytosol by a complex of heat-shock protein 90 and CAR cytoplasmic retention protein. Xenobiotics can activate CAR by both direct ligand binding and indirect protein modification mechanisms, causing dissociation from its chaperone proteins, followed by translocation to the nucleus (Mackowiak and Wang, 2016). Additionally, CAR is known to be inactivated by the androstane metabolites, androstenol and

androstanol, which act as inverse agonists (Kobayashi et al., 2015). Following translocation to the nucleus, CAR forms a heterodimer with the retinoid X receptor, binds to its response elements, and recruits co-activators SRC1 and GRIP1 to regulate target gene transcription (Mackowiak and Wang, 2016). CAR activation results in the differential regulation of over 2000 genes in mice and has overlap with pregnane X receptor and farnesoid X receptor (FXR, NR1H4) signaling (Cui and Klaassen, 2016). CAR activation is well known for mediating robust inductions of *CYP2B6* in humans and *Cyp2b10* in mice.

FXR is a NR that serves as a master regulator in bile acid (BA) homeostasis. BAs are amphipathic molecules that aid in the digestion and absorption of lipids and lipid-soluble vitamins in the small intestine. BAs are reabsorbed in the distal small bowel where they activate FXR to initiate a negative feedback mechanism, controlling their own synthesis (Makishima et al., 1999). Activation of intestinal FXR by BAs results in a strong induction of fibroblast growth factor 15 (FGF15) in mice and FGF19 in humans. FGF15/19 act as endocrine molecules, traveling to the liver where they interact with fibroblast growth factor receptor 4 (FGFR4) on hepatocytes. Activation of FGFR4 leads to the activation of the mitogen-activated protein kinases (MAPK) signaling pathway, including extracellular signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), to reduce BA production by suppressing the expression of genes involved in BA synthesis, including *CYP7A1/Cyp7a1* and *CYP8B1/Cyp8b1* (Kong et al., 2012).

Many liver diseases are initiated and/or worsened by BA dysregulation, including cholestasis, non-alcoholic fatty liver diseases (NAFLD), and liver tumors (hepatocellular carcinoma and cholangiocarcinoma). Nonalcoholic steatohepatitis (NASH) is within the more severe spectrum of NAFLD, which includes liver steatosis and inflammation. Dysregulations of many of the metabolic pathways governed by FGF15/19 have been found to be sequelae of NASH. NASH is rapidly becoming a major health issue affecting approximately 3-12% of the US population (Spengler and Loomba, 2015) and is projected to overtake hepatitis C virus as the leading indication of liver transplant in the United States (Noureddin et al., 2018). Currently there are no approved drug therapies for the treatment of NASH but synthetic FXR ligands and modified FGF19 proteins are in clinical trials for the treatment of NASH and cholestatic liver diseases.

As pharmaceutical companies continue to investigate the potential of FXR agonism and FGF19 therapies for disease intervention, it is important to understand the long-term effects of these therapies on liver function. The impact of sustained elevations of plasma FGF15/19 protein on xenobiotic metabolism is unknown. Alterations to DMETs can lead to drug-drug interactions, in which a perpetrator drug alters the disposition and/or action of a victim drug when taken in combination. Drug-drug interactions can lead to the loss of efficacy or toxicity in patients, therefore it is important to properly evaluate the potential of new therapies to alter DMETs. In the current study, using the *Fgf15* transgenic mice (*Fgf15* Tg) and intestine-specific *Fgf15* knockout mice (*Fgf15^{int}^{-/-}*), as well as *in vitro*

synthesized FGF19 protein, we have determined the effect of modulating FGF15/19 levels on the hepatic expression and function of DMET genes. Additionally, using *in vitro* primary human hepatocytes (PHH) and *in vivo* CAR knockout mice (CAR^{-/-}), we have examined the role of CAR in mediating the alterations of gene expression following FGF15/FGF19 overexpression.

Materials and Methods

Animals and Treatment. Male 8-12 week old wild type (WT), *Fgf15* Tg, and *Fgf15*^{int-/-} mice, all on C57BL/6J genetic background, were used (n=3-5). The generation of *Fgf15*^{int-/-} mice is detailed in **Supplemental Fig. 4** and **Supplemental Doc. 1**. *Car*^{-/-} mice on a mixed genetic background were generously gifted by Dr. Wen Xie from the University of Pittsburgh (Saini et al., 2004). F1 heterozygotes mice were obtained by crossing the *Car*^{-/-} mice with *Fgf15* Tg mice, then intercross breeding of F1 heterozygotes was adapted to obtain genetic background matched WT, *Car*^{-/-}, *Fgf15* Tg and *Car*^{-/-}/*Fgf15* Tg mice. Male WT, *Car*^{-/-}, *Fgf15* Tg and *Car*^{-/-}/*Fgf15* were 12-16 weeks of age at time of necropsy. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Rutgers University. For positive controls of *Cyp2b10* and *Cyp3a11* induction in the liver, WT mice were IP injected with phenobarbital (PB, 50 mg/kg) for 3 days, and the control group was treated with PBS. All mice were euthanized for tissue collection between 10:00-11:00 AM without fasting. Methods for serum biochemical assays and liver histopathological examination have been described previously (Schumacher et al., 2020). Additional animal information can be found in **Supplemental Fig. 1**

In vitro Treatment with Recombinant FGF19 Protein. HepaRG cells were cultured as previously described (Hart et al., 2010; Pande et al., 2020). PHHs were from a cryopreserved pool (5 donors) of human hepatocytes (PHH8007A, IVAL, Columbia, MD). PHH were plated into a 6- or 24-well collagen coated plates at a

density of 0.7×10^6 cells/ml and allowed to attach for 4 hours. Following attachment, plating medium was removed and the cells were changed to hepatocyte induction medium (HIM, IVAL, Columbia, MD). The plate was cultured in an incubator maintained with a humidified atmosphere of 95% air and 5% CO₂. HepaRG and PHH were treated with recombinant FGF19 (Kong and Guo, 2014) at concentrations of 5 or 50 ng/ml for 24 and 48 hours. Recombinant FGF19 was diluted in PBS and PBS was used as the vehicle control.

RNAseq Analysis. Total liver RNA from WT, *Fgf15* Tg, or *Fgf15*^{int/-} male mice (n= 3 per group) was extracted from frozen tissue by TRIzol method (Thermo Fisher Scientific, Waltham, MA). Whole-transcriptome cDNA libraries were prepared by NuGEN Mondrian system. Paired-end sequencing was performed using the Illumina HiSeq 2000 platforms (Illumina, Inc., San Diego, CA). Alignment and read quantification were performed for all samples and FPKM values for each liver sample were calculated based on the method for RNAseq data analysis described previously (Peng et al., 2012).

Gene Expression. Relative gene expression was determined as previously described (Rizzolo et al., 2019). Briefly, total RNA was extracted from frozen liver or ileum tissue using TRIzol reagent. Reverse transcription was performed to acquire cDNA. Real-time quantitative polymerase chain reaction (RT-PCR) was performed on the ViiA7 Real Time PCR Machine (Life Technologies, Grand Island, NY) using SYBR green to determine relative gene expression. Ct values were

converted to delta delta Ct values and normalized to β -actin. Primer sequences used in this study can be found in **Supplementary Fig. 1**.

Western Blot Analysis. Livers were homogenized and lysed in 1X radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA). Proteins (20 μ g per well) were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The blots were blocked for 2-hours at room temperature with 5% nonfat milk, incubated with primary antibody overnight at 4°C, incubated with species-specific secondary antibody for 1 hour at room temperature, and visualized using ECL substrates (Thermo Fisher Scientific, Waltham, MA). A list of antibodies used in this study can be found in **Supplemental Fig. 1**. Protein loading was normalized to levels of GAPDH or β -actin.

CYP2B and CYP3A Enzyme Activity. Liver microsomes were isolated from liver tissue, as previously described (Tien et al., 2015). Pentoxyresorufin and midazolam were used as probe substrates for the reactions pentoxyresorufin O-dealkylation and midazolam 1'-hydroxylation, which was used to detect the enzymatic activities of CYP2B and CYP3A, respectively. In brief, the incubation of 50 μ g of mouse liver microsomes was carried out in 1x phosphate-buffered saline (pH 7.4) with 30 μ M substrate concentration in a total volume of 95 μ L. The reactions were initiated with 5 μ L of 20 mM NADPH. Reactions containing pentoxyresorufin were carried out for 30 minutes and reactions containing midazolam were carried out for 10

minutes. All reactions were terminated by the addition of 100 μ L of ice-cold acetonitrile. The samples were vortexed for 30 seconds, centrifuged at 15,000 rpm for 10 minutes, and 1.0 μ L aliquots of the supernatant were injected into Waters Synapt G2-S QTOFMS system (Waters, Milford, MA) for metabolite analysis. Chromatographic separation of metabolites was performed on an Acquity UPLC BEH C18 column (2.1 $\times$ 100 mm, 1.7 μ m, Waters). Details on the analytical methodology can be found in **Supplemental Doc. 1**.

LC-MS/MS-based Protein Quantification. Mouse liver microsome samples were digested for LC-MS/MS-based proteomic analysis as previously described (Shi et al., 2018). 80 μ g of protein from liver microsomes were mixed with 0.2 μ g of BSA internal standard. A detailed description of sample preparation and digestion can be found in **Supplemental Doc. 1**. The digested samples were analyzed on a TripleTOF 5600+ mass spectrometer (AB Sciex, Farmingham, MA) coupled with an Eksigent 2D plus LC system (Eksigent Technologies, Dublin, CA). LC separation was performed via a trap-elute configuration including a trapping column (ChromXP C18-CL, 120 $\text{\AA}$, 5 μ m, 10 $\times$ 0.3 mm, Eksigent Technologies, Dublin, CA) and an analytical column (ChromXP C18-CL, 120 $\text{\AA}$, 150 $\times$ 0.3 mm, 5 μ m, Eksigent Technologies, Dublin, CA). A detailed description of the analytical methodology can be found in **Supplemental Doc. 1**. The surrogate peptides used for quantification of CYP2B9, CYP2B10, CYP2B19, CYP3A11, CYP3A13, CYP3A16, and CYP3A25 are listed in **Supplemental Table 1**. These peptides were selected based on their uniqueness and chromatographic performance. The peak areas of top 3 to 5 fragment ions were

summed up and normalized to the internal standard BSA. The BSA-normalized peak area of each peptide was further divided by the average of the 18 samples to calculate the relative abundance of the peptide. The average of relative abundance of all surrogate peptides of a protein was used to determine the relative abundance across different microsome samples.

CAR Nuclear Translocation by FGF19. Adenovirus expressing enhanced yellow fluorescent protein-tagged hCAR (Ad/EYFP-hCAR) was used in PHHs (Bioreclamation In Vitro Technologies, Baltimore, MD). PHHs with over 90% viability were seeded at 0.25×10^6 cells/well in 24-well biocoated plates in INVITROGRO™CP Medium (Bioreclamation In Vitro Technologies, Baltimore, MD) and infected with Ad/EYFP-hCAR (6 μ L/mL) as described previously (Li et al., 2009). Twenty-four hours after infection, PHHs were treated with vehicle control (0.1% DMSO), PB (1 mM), CITCO (1 μ M), or FGF19 (40 and 200 ng/mL) for 8h. EYFP-hCAR localization in hepatocytes was visualized on a Nikon Eclipse TI fluorescent microscope (Nikon, Melville, NY).

Body Composition CT Imaging. Male 5-month old WT and *Fgf15* Tg mice (n=5/group) were imaged using an Albira PET/CT (Bruker, Billerica, MA) as previously described (Metzinger et al., 2014; Murray et al., 2020). In brief, anesthetized mice were scanned using the following settings: Good Low High scan setting (400 slices), field of view (120mm²) with tube current (200 μ A) and voltage (45kV). After the scans were completed, they were reconstructed to a “Best” setting,

which increased the total slices to 600. The images were then analyzed using VivoQuant™ 2.0 imaging software. Hounsfield unit (HU) histograms were produced using 1000 bins describing a range of -1000 to 4000 HU. HU distribution was divided into three regions: fat (-350 to -75 HU), muscle (-75 to 200 HU), and bone (200 to 1000 HU). HU for each region was summed, divided by 512 (image matrix), corrected for bin total (1000), and then multiplied by tissue density factors of 0.9, 1.0, and 1.9 (g/cm³) for fat, muscle, and bone, respectively. To determine percent represented by each tissue, the previously described determination of mass was divided by the animal's body weight.

Pharmacokinetic Study. Male 4-month old WT and *Fgf15* Tg mice were injected I.V. with 1 mg/kg of bupropion (free base). Twenty µL of whole blood was collected with K₃EDTA at 3, 30, 60, 120, 180, and 240 minutes. Samples were stored on ice and spun for 10 minutes at 10,000g for plasma collection. 10 µL of mouse K₃EDTA plasma was spiked with 10 µL of a mixture of internal standards (250 ng/mL HBUP-D6 and 1000 ng/mL BUP-D9 in MeOH) and 10 µL of 10% trichloroacetic acid. Samples were vortexed for 5 min and centrifuged for 5 min at 10°C with 15 700 x *g*. The resulted supernatant was filtered through 0.2 µm PVDF filters at 10°C. The filtrate was then transferred to the HPLC vials with high-recovery inserts, and 10 µL was injected into the column. Bupropion and hydroxybupropion concentrations were determined by LC-MS/MS. A detailed description of the analytical methodology can be found in **Supplemental Doc. 1**. Pharmacokinetic parameters

were calculated by noncompartmental analysis with I.V. bolus dosing using PK Solver 2.0.

Serum GH and IGF-1 ELISA Measurements. Blood samples were collected every 30 mins from 9:30 am to 2:30 pm from WT and *Fgf15* Tg mice (male n = 5/genotype, female n = 3/genotype). In each collection, approximately 20 μ L of blood was collected from the lateral tail vein at each designated time point. After collection, blood samples were allowed to coagulate at room temperature for 10 mins, followed by centrifugation at 8000 g for 10 mins to prepare for serum. The serum samples were transferred to a fresh tube and stored at -80°C until assay. Commercially available ELISA kits for growth hormone (GH) (EZRMGH-45K, Millipore-Sigma) and insulin-like growth factor-1 (IGF-1) (DY791, R&D Systems) were used to determine their levels in the serum. In order to reduce inter-sample dilution effects, serum samples were diluted identically 2 and 500 times before performing GH and IGF-1 ELISA assays, respectively. The sensitivities for the ELISA assays are 70 pg/ml and 3.5 pg/ml for GH and IGF-1, respectively.

Statistical Analysis. The data are presented as mean $\pm$ 1 SD. Groups were compared using a one-way ANOVA followed by Tukey post-hoc unless otherwise noted. Data that failed assumptions of parametric statistics were run as Kruskal-Wallis. Statistical analysis was run using SAS Studio. Data were considered significant at P -values < 0.05 .

Results

Transcriptomes in FGF15 modified mouse livers

We have previously reported on the development of *Fgf15* Tg mice (Kong et al., 2018). The enterocyte-specific Fgf15 deficient mice (*Fgf15^{int-/-}*) were generated in our laboratory by the recombineering technology (**Supplemental Fig. 4 and Supplemental Doc. 1**). In **Fig. 1A**, qPCR confirmed the overexpression of *Fgf15* in both the liver and ileum. The protein levels of FGF15 in serum of *Fgf15* Tg mice were markedly increased, while serum FGF15 levels in WT mice were undetectable as measured by a commercially available ELISA kit (data not shown). As expected, the overexpression of *Fgf15* resulted in a significant reduction of *Cyp7a1* mRNA levels. This reduction is a result of functional FGF15 binding to FGFR4 and not a result of hepatic FXR activation as these mice have significantly reduced levels of BAs (Kong et al., 2018) and there was no change in the hepatic expression of *FXR* or a classical FXR target gene, *Shp* (*Nr0b2*). In order to further understand transcriptional regulation affected by FGF15, we profiled the liver transcriptomes of WT, *Fgf15* Tg and *Fgf15^{int-/-}* mice by RNA-seq analysis. Consistent with previous findings, the RNA-seq analysis (**Fig. 1B**) showed *Fgf15* Tg mice had reductions of the expression of hepatic genes involved in BA synthesis, such as *Cyp7a1* and *Cyp8b1*. *Fgf15^{int-/-}* mice displayed slight inductions of *Cyp7a1* and *Cyp8b1*, likely due to reduced FGF15-FGFR4 binding. Amongst genes that were induced in *Fgf15* Tg mice were critical genes involved in phase I drug metabolism, such as *Cyp2b10*, *Cyp2b9*, and *Cyp3a11*.

Gene and protein expression of hepatic drug metabolizing enzymes

RNA-seq analysis showed that the overexpression of *Fgf15* led to differential expression patterns for several cytochrome P450 genes involved in drug metabolism. **Fig. 2A** shows the validation of the observed induction of *Cyp2b9*, *Cyp2b10*, *Cyp3a11*, and *Cyp3a25* in *Fgf15* Tg mice at mRNA levels by RT-qPCR. Compared to WT mice, the mRNA levels of *Cyp2b9*, *Cyp2b10*, *Cyp3a11*, and *Cyp3a25* were all significantly increased in the livers of *Fgf15* Tg mice, with no significant changes to those in the livers of *Fgf15*^{int/-} mice. In order to determine if the observed alterations at the mRNA level were manifested at the protein level, we determined the protein expression of CYP2B and CYP3A in these mice by Western blots. Using antibodies targeting CYP2B and CYP3A, the results showed no change in the relative protein levels of CYP3A, an increase in CYP2B in *Fgf15* Tg mice but not in *Fgf15*^{int/-} mice, compared to WT mice (**Fig. 2B**).

Determine the protein isoform expression by LC-MS/MS-based proteomics.

Because Western blot analysis can be limited in its ability to differentiate between cytochrome P450 isoforms, we further determined the protein levels of individual CYP2B and CYP3A family members via LC-MS/MS. **Fig. 2C** shows the effects of FGF15 modulation on individual CYP2B and CYP3A isoforms in the liver. In male WT mice, the predominately expressed CYP2B family members are CYP2B10 and CYP2B19, while CYP2B9 is undetectable as CYP2B9 is predominantly expressed in female mice. However, *Fgf15* Tg male mice showed marked inductions of CYP2B9, as compared to WT. *Fgf15*^{int/-} mice generally had less CYP2B protein, regardless of

isoform. Male WT mice had relatively equal amounts of CYP3A11, CYP3A13, and CYP3A25 while displaying no CYP3A16, which is generally found in neonates and female mice. *Fgf15*^{int-/-} mice had minor reduction in CYP3A protein as compared to WT and also had no detectable CYP3A16. *Fgf15* Tg mice had about a 50% reduction in CYP3A11 protein despite showing an induction in *Cyp3a11* mRNA, as compared to WT mice. *Fgf15* Tg mice also had a slight reduction in CYP3A13 protein as compared to WT and a relatively similar amount of CYP3A25 protein. Interestingly, the *Fgf15* Tg mice did express CYP3A16 protein, which was not detected in the male WT mice.

Functional activity of CYP2B and CYP3A enzymes

The functional activities of CYP2B and CYP3A were measured by treating isolated microsomes with the CYP2B and CYP3A probe substrates pentoxyresorufin and midazolam, respectively. Specifically, we measured the reactions of pentoxyresorufin O-dealkylation and midazolam 1'-hydroxylation by LC/MS, which was used to assess the enzymatic activities of CYP2B and CYP3A, respectively. Microsomes isolated from *Fgf15* Tg mice displayed a 1.70 fold increase in resorufin metabolite formation as compared to WT mice, suggesting the observed induction in CYP2B protein and mRNA resulted in an increase of CYP2B metabolic rate ($P = 0.076$). Microsomes from *Fgf15*^{int-/-} mice showed no significant change from WT with a fold change of 0.93, as compared to WT (**Fig. 2D**). PB-treated mice were used as a positive control and displayed a 6.41 fold increase in pentoxyresorufin O-dealkylation. There was no observed difference in the rate of midazolam

hydroxylation among WT, *Fgf15*^{int/-}, and *Fgf15* Tg mice, suggesting there is no change to CYP3A hepatic microsomal activity.

FGF19 did not induce CYP2B and CYP3A in HepaRG or PHH cells

We have previously described the successful production of soluble recombinant FGF19 protein (Kong and Guo, 2014). We used the recombinant FGF19 protein to treat HepaRG and PHH cells at concentrations of 5 ng/mL and 50 ng/mL for 24 and 48 hours. Although FGF19 treatment led to activation of FGFR4 pathway (data not shown), it did not induce the mRNA expression of *CYP2B6* or *CYP3A4* regardless of the cell type, concentration and time point (**Supplemental Fig. 2**).

Effects of FGF19 on CAR nuclear translocation *in vitro*

CAR is a classical xenobiotic sensing NR known to regulate the expression of many DMET genes. In order to determine whether FGF19 was capable of activating CAR in PHHs, an adenovirus expressing enhanced yellow fluorescent protein tagged hCAR (Ad/EYFP-hCAR) was used. Through the use of fluorescent microscopy, we were able to visualize the localization of the Ad/EYFP-hCAR following treatment with known activators of CAR (PB and CITCO) or recombinant FGF19 protein.

Following treatment with PB (1mM) or CITCO (1 μ M), the Ad/EYFP-hCAR translocated from the cytosol (diffuse YFP seen in the vehicle control group) to the nucleus. While the FGF19 (40 ng/mL or 200 ng/mL) treated PHHs showed almost no nucleus translocation of the Ad/EYFP-hCAR, as compared to the PB and CITCO

treated groups (**Fig 3A**). These data suggested that recombinant FGF19 protein could not activate CAR *in vitro*.

Effects of CAR on FGF15-alteration of DMET gene expression *in vivo*

Although FGF19 did not directly activate CAR *in vitro*, indirect activation of CAR *in vivo* remains a valid hypothesis. In order to determine the *in vivo* role of CAR on the observed inductions of drug metabolism genes in *Fgf15* Tg mice, we crossed *CAR*^{-/-} mice with the *Fgf15* Tg mice to create *Fgf15* Tg/*CAR*^{-/-} mice. These mice develop and breed normally. Serum assays in WT, *Fgf15* Tg, *CAR*^{-/-}, and *Fgf15* Tg/*CAR*^{-/-} mice showed no significant differences between groups for total cholesterol, triglycerides, or ALT, AST, or ALP activities (**Supplemental Fig. 3**). Liver histology was examined by a board certified pathologist. All WT and *Fgf15* Tg mice had no noteworthy findings. Most *Fgf15* Tg/*CAR*^{-/-} mice displayed mild biliary hyperplasia. A detailed table of pathological findings can be found in **Supplemental Fig. 3**.

In **Fig. 3B** we measured the gene expression of 4 genes that are positively regulated by CAR activation (*Gsta1*, *Gstm3*, *Akr1b7*, *Cyp2b10*) and 3 genes that are negatively regulated by CAR activation (*Cyp2c55*, *Hsd3b5*, *Slco1a1*). Of genes positively regulated by CAR, *Fgf15* Tg mice displayed a 2.07 ± 1.17 and 1.93 ± 0.82 fold induction of *Gstm3* and *Akr1b7*, respectively, as compared to WT mice. *Fgf15* Tg/*CAR*^{-/-} mice showed a slightly greater induction in the expression of *Gstm3* and *Akr1b7*, with a significant fold change of 2.60 ± 1.45 and 2.58 ± 1.68 , respectively. *Cyp2b10*, a prototypical CAR target gene is significantly induced 12.46 ± 6.11 fold in

Fgf15 Tg mice, as compared to WT mice. In *CAR*^{-/-} mice, the expression of *Cyp2b10* was significantly reduced to 0.16 ± 0.16 . In *Fgf15* Tg/*CAR*^{-/-} mice *Cyp2b10* expression was significantly induced 3.70 ± 2.22 fold as compared to WT, which is significantly less than that of the *Fgf15* Tg mice ($P = 0.0004$). For genes negatively regulated by CAR activation, there was no significant difference in the expression of *Hsd3b5* or *Slco1a1* between WT and *CAR*^{-/-} mice. However, *Fgf15* Tg mice displayed a significant reduction in *Hsd3b5* and *Slco1a1* mRNA expression of 0.18 ± 0.36 and 0.24 ± 0.27 , respectively. This reduction was further exacerbated in *Fgf15* Tg/*CAR*^{-/-} mice to 0.02 ± 0.02 and 0.09 ± 0.07 , respectively, as compared to WT mice. In **Fig. 3C**, we measured the mRNA expression of 6 genes involved in phase I or phase II drug metabolism (*Cyp1a*, *Cyp3a11*, *Cyp3a16*, *Cyp3a44*, *Ugt1a1*, *Sult1a1*). There was no significant difference for any of the genes measured among WT, *CAR*^{-/-}, *Fgf15* Tg, and *Fgf15* Tg/*CAR*^{-/-} mice.

Amongst the differentially expressed cytochrome P450s described in **Figs. 1 & 2**, were *Cyp2b9* and *Cyp3a16*, which are known to have sexually dimorphic expression patterns, with both being expressed higher in female than in male mice. Using male WT, *Fgf15* Tg, *CAR*^{-/-}, and *Fgf15* Tg/*CAR*^{-/-} mice, we measured the relative expression of 6 genes that are predominantly expressed in male mice (**Fig. 3D**; *Hsd3b5*, *Ugt2b1*, *Srd5a1*, *Slco1a1*, *Mup1*, *Cyp2d9*) and the relative gene expression of 5 genes that are predominantly expressed in female mice (**Fig. 3E**; *Cyp2b9*, *Cyp2b13*, *Cyp17a1*, *Sult1e1*, *Hsd3b1*). *Fgf15* Tg mice displayed significant reductions in the expression of male dominant genes (**Fig. 3D**) *Hsd3b5*, *Ugt2b1*, *Slco1a1*, *Mup1*, and *Cyp2d9*, with

a trend towards a significant reduction of *Srd5a1* ($P=0.0686$). There was no significant difference between WT and *CAR*^{-/-} mice for any of the genes measured; however in *Fgf15* Tg/*CAR*^{-/-} mice the significant reductions of male dominant genes in *Fgf15* Tg mice was further exacerbated in the absence of *CAR*. Male *Fgf15* Tg and *Fgf15* Tg/*CAR*^{-/-} mice both displayed significant increases in the expression of *Cyp2b9*, *Cyp2b13*, and *Cyp17a1* with trends for increases in *Sult1e1* and *Hsd3b1* (**Fig. 3E**), genes that are predominantly expressed in female mice.

The impact of the overexpression of *Fgf15* on the pharmacokinetics of bupropion

To assess the functional implications of the induction of CYP2B mRNA and protein in the *Fgf15* Tg mice, we performed a pharmacokinetic study using bupropion as a probe substrate to measure CYP2B activity *in vivo*. WT and *Fgf15* Tg mice were dosed with 1 mg/kg of bupropion I.V., plasma concentrations of bupropion and the metabolite hydroxybupropion were taken at 3, 30, 60, 120, 180, and 240 minutes. The mean plasma (nM) semi-logarithmic concentration time plots are shown in **Fig. 4A**. A summary table (**Fig. 4B**) shows that *Fgf15* Tg mice have a reduction ($P=0.163$) in terminal half-life from 55.8 minutes to 46.8 minutes, as compared to WT. Additionally, *Fgf15* Tg mice displayed a higher C_{max} of hydroxybupropion (18.1 ng/mL) than WT mice (13.4 ng/mL) mice and *Fgf15* Tg mice reached that maximum metabolite concentration 27.4 minutes faster ($P=0.077$) than WT mice (a reduction of 28.5%). Taken together, these data suggest that *Fgf15* Tg mice convert

bupropion, a CYP2B probe substrate, to the metabolite hydroxybupropion at a faster rate than WT mice.

Body size and composition

Gross observations suggested differences in the size of WT and *Fgf15* Tg mice (**Fig. 5A**). *Fgf15* Tg mice had a significantly shorter body length measured by nose-to-anus length ($7.83 \pm 0.41\text{cm}$) than WT mice ($9.15 \pm 0.42\text{cm}$). *Fgf15* Tg mice also weighed significantly less ($22.45 \pm 2.18\text{g}$) than WT mice ($27.98 \pm 0.94\text{g}$) (**Fig. 5B**). CT imaging was used to determine if the overexpression of *Fgf15* altered the disposition of fat, muscle, or bone (**Fig. 5C**). There were no noteworthy alterations in the distribution of fat, muscle or bone between WT mice (6.9%, 76.2%, and 16.0% respectively) and *Fgf15* Tg mice (7.4%, 74.0%, and 18.0% respectively).

Cell signaling and growth hormone signaling pathways

FGFR4 activation by FGF15/19 is known to activate several intracellular signaling pathways, including ERK1/2-MAPK, PI3K-AKT, and JAK/STAT pathways (Liu et al., 2020). In **Fig. 5D**, we measured the modification of these pathways and semi-quantified the Western blot analysis. We observed an overall reduction in the phosphorylation of AKT, ERK, STAT3, and STAT5 in *Fgf15* Tg mice, as compared to WT. The reduction may be a result of desensitization to FGFR4 activation as a result of the continuous overexpression of *Fgf15*. Of note is the drastic reduction of phosphorylated STAT5 in 3 out of 4 *Fgf15* Tg samples measured. STAT5 signaling is well known to be activated by growth hormone and influence sexually dimorphic

hepatic gene expression patterns and growth development (Zhang et al., 2012; Martinez et al., 2013). Measurements of the mRNA levels of GH response genes, *Igf-1* and *Igfals*, showed reductions of gene expression in the livers of *Fgf15* Tg mice ($P=0.068$ and 0.085 , respectively), as compared to WT mice (**Fig. 5E**).

Serum Levels of GH and IGF-1

In order to assess potential differences in GH release between WT and *Fgf15* Tg mice, we collected blood from mice every 30 minutes from 9:30am to 2:30pm. In **Fig. 5F**, we measured the serum levels of GH and the GH response gene, IGF-1, via ELISA assays. WT mice generally displayed a typical male GH release pattern with a robust GH pulse beginning around 12:00 pm and reaching a maximum concentration around 12:30 pm. *Fgf15* Tg mice showed a GH release pattern more similar to female mice with low, steady levels detected. The serum GH levels for female mice were similar between WT and *Fgf15* Tg mice, with levels generally being maintained between 2-4 ng/mL (**Supplemental Fig. 5A**). Serum IGF-1 concentrations were more varied but WT mice tended to have higher levels of serum IGF-1 than *Fgf15* Tg mice.

Discussion

FGF15 and its human orthologue FGF19 are endocrine FGFs that function to suppress BA production, reduce steatosis, regulate oxidative stress, promote liver growth and protein production, and improve insulin resistance in murine models of NASH (Henriksson and Andersen, 2020; Stofan and Guo, 2020). As such, FXR

agonists, which are capable of producing robust inductions of FGF15/19 through ileal FXR activation, as well as FGF19 analogs/mimetics, have been in clinical trials for the treatments of NASH and cholestatic liver diseases. In the present study, we determine the effects of FGF15/19 overexpression on DMETs *in vivo*, through the use of an *Fgf15* Tg mouse model, and *in vitro*, using recombinant FGF19 protein.

The overexpression of *Fgf15* caused a broad alteration of the expression of DMETs revealed by RNA-seq transcriptomic analysis. Alterations of DMETs can result in unintended clinical consequences, such as loss of efficacy or toxicity. For this reason, we wanted to explore the functional consequences of these alterations, as well as investigate the potential mechanisms underlying this regulation. Among the genes with their expression altered, we confirmed inductions of *Cyp2b9*, *Cyp2b10*, *Cyp3a11* and *Cyp3a25* at the mRNA level and inductions of CYP2B9, CYP2B10, CYP2B19, and CYP3A16 at the protein level as a result of the overexpression of *Fgf15*. Through the use of isolated hepatic microsomes from *Fgf15* Tg mice, we found an increase in the metabolism of pentoxoresorufin ($P=0.076$), a probe substrate for CYP2B activity. Our pharmacokinetic study also found an increase ($P=0.163$) in the rate of metabolism of bupropion, a CYP2B specific substrate, to the metabolite hydroxybupropion by *Fgf15* Tg mice, as compared to WT mice.

CAR is a well-studied xenobiotic sensing NR known to induce human CYP2B6 and CYP3A4 (CYP2B10 and CYP3A11 in mice). Recent research has shown a connection between FGF15 and FXR or CAR activation in a tissue specific manner (Weber et al.,

2021). In order to determine if the observed alterations in *Fgf15* Tg mice were a result of elevated CAR activation, we crossbred *Fgf15* Tg mice with *CAR*^{-/-} mice to create *Fgf15* Tg/*CAR*^{-/-} mice. We found that in some cases (*Gstm3* and *Akr1b7*), the loss of *CAR* exacerbated inductions brought about by the overexpression of *Fgf15*. In other cases (*Cyp2b10* and *Cyp17a1*), the loss of *CAR* attenuated inductions caused by the overexpression of *Fgf15*. Taken together, these data suggest *CAR* may play a role in altering the expression pattern of DMETs in mice overexpressing *Fgf15*; however, there are clearly additional mechanisms driving the observed alterations.

Amongst the phase I metabolizing enzymes that were induced in male *Fgf15* Tg mice was *Cyp2b9*. *Cyp2b9* is a sexually dimorphic cytochrome P450 that is predominantly expressed in female mice (Wiwi et al., 2004). Looking at other hepatic genes known to be sexually dimorphic, we found that male *Fgf15* Tg mice had large inductions of genes primarily expressed in the livers of female mice, e.g., *Cyp2b9*, *Cyp2b13*, *Cyp17a1*, *Sult1e1*, *Hsd3b1*. Conversely, we looked at the hepatic mRNA expression of male dominant genes and found that male *Fgf15* Tg mice had a significantly lower expression pattern of many of these genes, including *Hsd3b5*, *Ugt2b1*, *Slco1a1*, *Mup1*, and *Cyp2d9*.

STAT5 is a transcription factor activated predominately by growth hormone and plays a key role in the sexually dimorphic expression of cytochrome P450s in the liver (Davey et al., 1999). Neuroendocrine factors regulate a pulsatile (in male) or continual (in female) GH release, which, amongst other things, regulate the tyrosine

phosphorylation of STAT5b in the liver. In general, male mice go through cyclical periods of robust STAT5b phosphorylation/activation with periods of little or no activated STAT5b during the GH interpulse interval. Female mice have continual, low (but measurable) plasma GH and STAT5b activity (Waxman and O'Connor, 2006). Additionally, GH is known to influence body size and weight in mice (Kopchick et al., 2014). In line with this known mechanism of sexual dimorphism, our male *Fgf15* Tg mice exhibited a low consistent concentration of serum GH, while our male WT mice displayed the prototypical male GH surge. As a likely result of altered GH signaling, our *Fgf15* Tg mice had greatly reduced STAT5 phosphorylation as compared to WT mice. Downstream measurements of GH response genes *Igf-1* and *Igfals* showed reduced mRNA expression in the livers of *Fgf15* Tg mice ($P=0.068$ and 0.085 , respectively). Additionally, *Fgf15* Tg mice have a shorter nose to anus length and reduced body weight compared to WT mice. Factors known to impact GH secretion patterns include stress, exercise, nutritional state, and metabolic signals (Kato et al., 2002). The observed alterations could be a result of reduced BAs impacting lipid and lipid-soluble vitamin absorption, causing changes to the nutritional state or metabolic signaling in the *Fgf15* Tg mice. The impact of FGF15/19 on GH regulation and STAT5b activity is the subject of an ongoing investigation in our lab.

The influence of FXR and BAs in liver disease etiology is well established. As pharmaceutical companies continue to probe the FXR-BA-FGF15/19 pathway for the therapeutic intervention in treating diseases associated with BA dysregulation,

it is important to understand the potential that manipulating these pathways have on the regulation of drug metabolizing enzymes. Through the use of an *Fgf15* Tg mouse model, we have shown that the overexpression of *Fgf15* induces the expression of several phase I metabolizing enzymes and leads to a phenotypical switch from a male to female expression pattern in the livers of male *Fgf15* Tg mice. The mechanism underlying this gender switch is unclear. However, the *Fgf15* Tg mice have much lower BA contents than WT mice, which negatively affect lipid absorption. Lower lipid absorption is a form a nutrient deprivation known to be associated with growth retardation. In our study, we found shorter body length, reduced STAT5 activation, altered serum GH levels, and reduced GH target gene expression (*Igf-1* and *Igfals*) as a result of *Fgf15* overexpression. GH reduction may lead to CAR activation by releasing factors known to inhibit CAR activation, including EGFR signaling and production of CAR endogenous inhibitors. We will further determine the underling mechanism in future studies to determine the extent to which reduced GH and STAT5 signaling is responsible for the observed gender specific switch of DMET expression.

In summary, we have shown that overexpression of FGF15 led to a gender specific switch of the expression of genes encoding DMETs in the liver. The mechanism responsible for this switch is unclear; however, initial data from the studies suggest that reduced GH signaling and increased CAR activation are associated with this switch.

Figure Captions

Fig. 1 Relative hepatic and ileal mRNA values of Fgf15 related genes and RNAseq analysis. (A) Hepatic and ileal gene expression was normalized to β -actin mRNA expression and graphs depict relative mRNA $\pm$ 1 SD (n: WT= 9, *Fgf15*^{int-/-}= 6, *Fgf15* Tg= 5; one-way ANOVA, Tukey post-hoc). An asterisk denotes a significant difference from WT ($P < 0.05$). (B) Heat map illustrates log2 fold-change in RNAseq analysis as compared to WT (n=3/group).

Fig. 2 Relative hepatic mRNA, protein, and function of cytochrome P450s. (A) Relative hepatic mRNA expression normalized to β -actin mRNA expression (n: WT= 5, *Fgf15*^{int-/-}= 6, *Fgf15* Tg= 5; one-way ANOVA, Tukey post-hoc). Graphs depict relative mRNA $\pm$ 1 SD. An asterisk denotes a significant difference from WT ($P < 0.05$). *Cyp2b9* and *Cyp2b10* failed Levene's test therefore Kruskal-Wallis was used for analysis. (B) Western blot of CYP3A and CYP2B using 20 μ g of liver homogenate (n: WT= 4, *Fgf15*^{int-/-}= 5, *Fgf15* Tg= 5; one-way ANOVA, Tukey post-hoc). (C) LCMS analysis of relative protein expression of CYP2B and CYP3A isoforms (n: WT= 5, *Fgf15*^{int-/-}= 5, *Fgf15* Tg= 5, WT-PB= 3; one-way ANOVA, Tukey post-hoc). . (D) Measurements of pentoxyresorufin and midazolam metabolite formation to assess CYP2B and CYP3A activity in liver microsomes of WT, *Fgf15*^{int-/-}, *Fgf15* Tg, and phenobarbital treated WT mice (n: WT= 5, *Fgf15*^{int-/-}= 5, *Fgf15* Tg= 5, WT-PB= 3; one-way ANOVA, Tukey post-hoc).

Fig. 3 Role of CAR in alterations brought about by the overexpression of *Fgf15/19*. (A) Fluorescent imaging of Ad/EYFP-hCAR PHHs treated with PB, CITCO, or FGF19 recombinant protein. (B-E) Hepatic gene expression was normalized to β -actin mRNA expression and graphs depict relative mRNA $\pm$ 1 SD. An asterisk denotes a significant difference from WT ($P < 0.05$). *Cyp2b10*, *Cyp2b9*, *Cyp2b13*, and *Cyp17a1* failed Levene's test therefore Kruskal-Wallis was used for analysis. (B) Relative hepatic mRNA expression of genes regulated by CAR. (C) Relative hepatic mRNA expression of genes involved in drug metabolism. (D) Relative hepatic mRNA expression of genes predominantly expressed in male mice. (E) Relative hepatic mRNA expression of genes predominantly expressed in female mice (n: WT= 6, *Fgf15* Tg= 7, CAR^{-/-}= 6, *Fgf15* Tg/CAR^{-/-}= 7; one-way ANOVA, Tukey post-hoc).

Fig. 4 Impact of *Fgf15* overexpression on the pharmacokinetics of bupropion in mouse plasma. (A) Average log concentrations of bupropion and hydroxybupropion (+SD) in wild type and *Fgf15* Tg mouse plasma. (B) Table summarizing pharmacokinetic parameters ($\pm$ SD) of bupropion and hydroxybupropion in wild type and *Fgf15* Tg mouse plasma (n: WT= 10, *Fgf15* Tg= 8; Student's *t*-test).

Fig. 5 Body size and composition of WT and *Fgf15* Tg mice. (A) Representative dorsal view of WT (top) and *Fgf15* Tg (bottom) mice. (B) Quantification of nose to anus length (cm) and body weight (g) of WT and *Fgf15* Tg mice (n: WT= 5, *Fgf15* Tg= 6; Student's *t*-test). (C) Percent of total body weight distributed as fat, muscle, and

bone (n: WT= 5, *Fgf15* Tg= 5; Student's *t*-test). (D) Western blots and semiquantifications of cell signaling proteins in liver homogenates from WT and *Fgf15* Tg mice (n: WT= 4, *Fgf15* Tg= 4). (E) Relative hepatic gene expression of GH response genes *Igf-1* and *Igfals* (n: WT= 6, *Fgf15* Tg= 7; Student's *t*-test). (F) Serum GH (left) and IGF-1 (right) levels in WT and *Fgf15* Tg mice measured by ELISA every 30 minutes from 9:30am to 2:30pm.

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Authorship Contributions

Participated in research design: Rizzolo, Kong, Guo

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Contributed new reagents or analytical tools: Kagan, Guo

Performed data analysis: Rizzolo, Kong, Piekos, Siemiątkowska, Zhu, Zhong, Wang, Guo

Wrote or contributed to the writing of the manuscript: Rizzolo, Guo

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Footnotes

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The authors declare that they have no conflicts of interest with the contents of this article.

Figure 1

Figure 2

Figure 3

Figure 4

B.

Pharmacokinetic parameter	Bupropion		Hydroxybupropion	
	Wild type	<i>Fgf15 Tg</i>	Wild type	<i>Fgf15 Tg</i>
AUC_(0-inf)(ng/ml * min)	23,320 ± 8,756	24,946 ± 15,968	3,992 ± 2,878	3,816 ± 3,055
C_{max} (ng/ml)	552.5 ± 127.2	531.6 ± 254.3	13.4 ± 4.1	18.1 ± 12.6
T_{max} (min)	3.0 ± 0.0	3.0 ± 0.0	96.0 ± 31.0	68.6 ± 22.7
T_{1/2} (min)	55.8 ± 21.5	46.8 ± 11.3	157.9 ± 113.4	105.3 ± 54.4
MRT_(0-inf)(min)	50.6 ± 18.6	50.1 ± 25.1	271.8 ± 167.5	194.3 ± 77.5

Figure 5