UP-REGULATION OF FIBROBLAST GROWTH FACTOR (FGF) 21 VIA AP-1 ACTIVATION ATTENUATES CISPLATIN-INDUCED HEPATOTOXICITY

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ABSTRACT

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Fibroblast growth factor (Fgf in rodents and FGF in human) 21 plays important roles in the maintenance of sugar, lipid and energy homeostasis. Fgf/FGF21 can be up-regulated via activation of peroxisome proliferator-activated receptors (PPARs), aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR), and activating transcription factor (ATF) 4. Recent studies also demonstrated that Fgf/FGF21 plays cytoprotective roles against chemical-induced toxicities, such as of dioxins, acetaminophen, and alcohols. Cisplatin (cis-diaminedichloroplatinum, CDDP) is a widely used chemotherapeutic drug. However, numerous adverse effects have been noted during CDDP therapy, which largely limit its clinical applications. This study was designed to determine the regulation of Fgf/FGF21 expression by CDDP, and to characterize the underlying mechanisms of its regulation, as well as to determine the significance of gain of Fgf/FGF21 function in attenuating CDDP-induced liver injury. Our results showed that CDDP induced mRNA and protein expression of Fgf/FGF21 in mouse livers as well as in cultured mouse and human hepatoma cells. In addition, CDDP activated activator protein-1 (AP)-1 but not ATF4 or Nrf2 signaling in mouse livers. We further demonstrated that the AP-1 activation is responsible for CDDP-induced Fgf/FGF21 expression. Furthermore, CDDP produces more severe liver injury and inflammation in Fgf21-null than wild-type mice. Pre-treatment of dexamethasone (DEX) or β-Naphthoflavone (BNF),
which induces Fgf21 expression, attenuated CDDP-induced hepatotoxicity. In conclusion, CDDP induced Fgf/FGF21 expression in mouse liver and mouse/human hepatoma cells via AP-1 activation. In addition, gain of Fgf/FGF21 function plays protective roles against CDDP-induced hepatotoxicity.
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AhR, aryl hydrocarbon receptor

ABCG, ATP-binding cassette G

ALT, alanine aminotransferase

AP-1, activator protein-1

APAP, acetaminophen

ATF4, activating transcription factor 4

AST, aspartate aminotransferase

BNF, β-naphthoflavone

BSEP, Bile salt export pump

CDDP, cis-diaminedichloroplatinum (II); cisplatin

ChIP, chromatin immunoprecipitation assay

DEX, dexamethasone
eIF2, eukaryotic initiation factor 2

FCCP, carbonyl-cyanide-4-(trifluoromethoxy) phenyhydrazone

FGF, fibroblast growth factor

FGFR, fibroblast growth factor receptor

GDF15, growth differentiation factor 15

HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase

HMGCS, 3-hydroxy-3-methylglutaryl coenzyme A synthase
HSGAGs, heparin or heparan sulphate glycosaminoglycans

JNK, c-Jun N-terminal kinases

KLB, β-klotho

Nrf2, nuclear factor erythroid 2-related factor 2

NTCP, Na⁺-taurocholate cotransporting polypeptide

PGC, peroxisome proliferator-activated receptor gamma coactivator

PPAR, peroxisome proliferator-activated receptor

qRT-PCR, quantitative real-time polymerase chain reaction

SHP, small heterodimer partner

SREBP, sterol regulatory element binding protein

SREBF, sterol regulatory element binding transcription factor

TG, triglycerides

TPA, 12-O-tetradecanoylphorbol-13-acetate

TSS, transcription start site

T2D, type 2 diabetes

WAT, white adipose tissue
CHAPTER 1 BACKGROUND AND INTRODUCTION

1.1 Overview

Fibroblast growth factor (Fgf in rodents and FGF in human) 21, an endocrine hormone, plays important roles in regulation of metabolic pathways and is commonly recognized as a novel target for managing metabolic syndrome. Metabolic syndrome, which is characterized by hypertension, hyperglycemia and dyslipidemia, increased risk of diseases such as type 2 diabetes (T2D), cardiovascular disease, nonalcoholic fatty liver disease (NAFLD), and certain cancers. Hirode et al. reported a prevalence of 34.7% in adults suffering from metabolic syndrome during 2011 to 2015. The prevalence significantly increased to 48.6% among those aged above 60 years (Hirode et al., 2020). The mechanisms by which FGF21 attenuates metabolic syndrome and stress have been extensively studied (Kim et al., 2014; BonDurant et al., 2018).

Fgf/FGF21 expression can be up-regulated through multiple signaling pathways, such as activation of peroxisome proliferator-activated receptors (PPARs), farnesoid X receptor (FXR), aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR), and activating transcription factor (ATF) 4 (Lundåsen et al., 2007; Cyphert et al., 2012; Cheng et al., 2014; Patel et al., 2015; Alonge et al., 2017). Recent studies also demonstrated that FGF21 plays cytoprotective roles against chemical-induced toxicities, such as of dioxins, acetaminophen, and alcohols (Cheng et al., 2014; Ye, D et al., 2014; Desai et al., 2017). It is currently noted that drug-induced toxicities in liver, kidney, heart
and brain account for more than 70% of drug attrition and drug withdrawal (Wilke et al., 2007).

Cisplatin (cis-diaminedichloroplatinum, CDDP) is a widely used chemotherapeutic drug. Numerous adverse effects, such as kidney and liver injury, have been noted during CDDP therapy, which largely limit its clinical applications (Oun et al., 2018).

The research project in my dissertation was designed to determine the regulation of mouse and human Fgf/FGF21 expression by CDDP, and the underlying mechanisms, as well as the impact of Fgf/FGF21 on the progression of CDDP-induced liver injury. Chapter 1 reviews the current literature on FGF superfamily, and an overview of pharmacology and physiology of FGF21, including its cytoprotective roles and therapeutic potential against metabolic stress, inflammation and tissue damage; as well as CDDP induced liver and kidney toxicities. The signaling pathway of activator protein (AP)-1 is also briefly discussed. Chapter 2 describes methodology and experimental design. Chapter 3 characterizes the underlying mechanism of CDDP-induced Fgf/FGF21 expression and the protective role of Fgf/FGF21 attenuating the progression of CDDP-induced liver injury. Chapter 4 discusses our results. Chapter 5 summarizes the overall and significant findings in my dissertation work. The potential future directions are identified and discussed as well.
1.2 Fibroblast Growth Factor (Fgf/FGF) Family

The fibroblast growth factor (Fgf/FGF) family is composed of totally 22 members that are involved in diverse biological functions, including cell growth and differentiation, angiogenesis, embryonic development, wound healing and repair, as well as metabolic regulation (Beenken et al., 2009). FGFs were classified based on sequence homology and phylogeny, in which 18 mammalian fibroblast growth factors (FGF1- FGF10 and FGF16- FGF23) are grouped into 6 subfamilies: FGF1 subfamily (FGF1 and FGF2); FGF4 subfamily (FGF4, FGF5 and FGF6); FGF7 subfamily (FGF3, FGF7, FGF10, FGF22); FGF8 subfamily (FGF8, FGF17 and FGF18); FGF9 subfamily (FGF9, FGF16 and FGF20); and Fgf15/FGF19 subfamily (Fgf15/FGF19, FGF21 and FGF23) (Itoh et al., 2004). Fgf15 is the mouse orthologue of human FGF19. Members of first five subfamilies, also named canonical FGFs, are autocrine and paracrine factors and act through tyrosine kinase FGF receptors (FGFR1-4), via a high-affinity interaction with heparin or heparan sulphate glycosaminoglycans (HSGAGs) on extracellular matrix followed by FGFR activation, dimerization and then activate downstream signal transduction pathways (Ornitz et al., 1996; Ornitz et al., 2000; Itoh et al., 2008). In contrast, members of Fgf15/FGF19 subfamily, including Fgf15/FGF19, FGF21 and FGF23, are endocrine FGFs and act as circulating hormones (Itoh et al., 2010), they lack a classic heparin binding domain and bind to FGFRs with low affinity even in the
presence of HSGAGs in extracellular matrix (Zhang et al., 2006). This situation is
compensated by the presence of α- and β-Klotho co-receptors, several transmembrane
glycoproteins, which dimerizes with and facilitates to activate their cognate FGFRs. α-
Klotho is a co-receptor for FGF23; whereas β-Klotho (KLB) is required for Fgf15/FGF19
and FGF21 (Ogawa et al., 2007; Urakawa et al., 2006; Wu et al., 2007; Agrawal et al.,
2018).

FGFRs include four members (FGFR1-FGFR4). Each FGFR protein consists of three
extracellular immunoglobulin domains (D1-D3), a single transmembrane domain, and a
cytoplasmic tyrosine kinase domain (Figure 1.1) (Beenken et al., 2009). FGFR1, -2, and -
3 have two alternative exons (b and c), which encode the second half of extracellular
immunoglobulin domains D3 (Fon Tacer et al., 2010; Tiong et al., 2013). The alternative
splice isoforms are generally tissue-specific: the b isoform is usually expressed in
epithelial tissue; whereas the c isoform is usually expressed in mesenchymal tissue
(Beenken et al., 2009). Most tissues express one or several isoforms. FGFR1, FGFR3,
FGFR4 and a minimal FGFR2 expression were observed in normal human hepatocytes
and bile duct epithelium (Hughes et al., 1997). In mice, FGFR1 and FGFR4 are
predominantly expressed in white adipose tissue and liver, respectively (Kurosu et al.,
2007). Therefore, target organs of the circulating Fgf15/FGF19 subfamily hormones can
be restricted by the unique tissue expression of α- and β-Klotho co-receptors (Urakawa et
al., 2006; Kurosu et al., 2007; Yang et al., 2012). α-Klotho is predominantly expressed in
the kidney and brain. KLB is more restricted to liver and adipose tissues, but is also found in the kidney, gut, and spleen (Kurosu et al., 2007; Fon Tacer et al., 2010; Zou et al., 2018). Endocrine FGFs, acting together with α- and β-Klotho proteins, have been studied to regulate glucose, cholesterol, and bile acid metabolism (Tomlinson et al., 2002; Beenken et al., 2009; Chen, M et al., 2018).

Figure 1.1 The basic structure of FGFRs.

The FGFRs consist of three extracellular immunoglobulin (Ig) domains (D1-D3), a single transmembrane helix, an intracellular split tyrosine kinase domain (TK1 and TK2), and an acidic box.

1.3 Fibroblast Growth Factor (FGF) 21

FGF21 is a member of endocrine FGF19 subfamily, which includes Fgf15/FGF19, FGF21 and FGF23. FGF21 shares 10-30% sequence similarity with other FGFs. However, FGF21 does not promote cell growth in vivo (Kharitonenkov et al., 2011). Its deficiency in heparin binding domain leads to a low binding affinity to extracellular matrix in which heparin/heparan sulphate glycosaminoglycans exist that consequently
lower local binding to its receptor FGFRs (Zhang et al., 2006; Kharitonenkov et al., 2011). Low binding affinity of FGF21 to its receptor can be compensated by the presence of a transmembrane β-Klotho (KLB) co-receptor, which dimerizes with and facilitates to activate the FGFRs (Degirolamo et al, 2016).

Fgf21 was initially isolated from mouse liver (Nishimura et al., 2000). In mice, Fgf21 is predominantly expressed in the liver and adipose tissues, and at much lower levels in heart, kidney and skeletal muscle (Muise et al., 2008; Planavila et al., 2015; Li, F et al., 2018). Whereas in humans, FGF21 is almost exclusively expressed in the liver (Shan et al. 2018). The actions of FGF21 appear to be mainly mediated by FGFR1c (Nies et al., 2016). Blunted systemic effects of FGF21 have been observed in Fgfr1-knockout mice (Foltz et al., 2012; Adams et al., 2013). It remains still unknown whether other FGFRs can mediate the functions of FGF21. In mouse liver, both Fgf19 and Fgf21 bind to Fgfr4/Klb complex; however, only Fgf19, but not Fgf21 can activate downstream phosphorylation of ERK signaling through Fgfr4 (Kurosu et al., 2007). In addition, the regulatory effects of FGF21 on cholesterol metabolism have been suggested that at least partly mediated by the FGFR2/KLB complex (Lin et al., 2015).

Fgf21 was first identified as a metabolic factor because it reduces plasma glucose and triglycerides to near normal levels in both obese and diabetic mouse models (Kharitonenkov et al., 2005). In both mouse and human, obesity and diabetes can induce Fgf/FGF21 expression in the liver via activation of Peroxisome Proliferator-Activated
Receptor (PPAR) α, and consequently leads to a PPAR-γ coactivator protein (PGC) 1α-dependent upregulation of fatty acid oxidation and gluconeogenesis but downregulation of lipid synthesis (Potthoff et al., 2009; Domouzoglou et al., 2015). Secreted FGF21 circulates through whole-body and acts on white adipose tissue (WAT), brown adipose tissue (BAT), and liver to promote the expression of genes that regulate sugar and lipid metabolism (Badman et al., 2007; Lundåsen et al., 2007; Kharitonenkov et al., 2009; Potthoff et al., 2009). A recent study demonstrated a direct effect of FGF21 on the ventromedial hypothalamus to enhance the sensitivity to glucose and lower sugar intake in mice, but with no apparent insulin sensitivity improvement, body weight reduction, or other metabolic parameters alterations (Jensen-Cody et al., 2020).

FGF21 shows potential therapeutic effects by reducing adiposity, ameliorating hyperglycemia and hyperinsulinemia, improving insulin resistance and dyslipidemia, as well as attenuating the progression of fatty liver (Kharitonenkov et al., 2007; Coskun et al., 2008). For instance, FGF21 analogs, such as LY2405319, PF-05231023 (a long-acting FGF21 analog) and Pegbelfermin (BMS- 986036, a PEGylated human FGF21 analog), improve dyslipidemia and plasma lipoprotein profile, with significant reduction of plasma triglycerides and total cholesterol in patients with T2D. However, their effects on glucose control are modest in the clinical trials (Gaich et al., 2013; Dong, J et al., 2015; Talukdar et al., 2016; Charles et al., 2019). The absence of glycemic response of
FGF21 therapy in clinical studies suggested a poor translation from rodents to humans in glucose-lowering effects (Kharitonenkov et al., 2015).

Metabolic diseases are often associated with endoplasmic reticulum (ER) stress and/or mitochondrial stress. Stress can lead to inflammation responses that result in apoptosis and even chronic pathologies such as fibrosis and cancers (Gómez-Sámano et al., 2017). FGF21 induction by different kinds of stress attenuates or prevents tissue from structural damage and functional deterioration via inhibiting inflammation (Luo et al., 2017). One of common mechanisms contributing to stress-induced FGF21 expression is via activation of the eukaryotic initiation factor 2α - activating transcription factor 4 (eIF2-ATF4) axis (Sousa-Coelho et al., 2012; Wan et al., 2014; Salminen et al., 2017). In addition, KLB expression can also be induced by ER stress via the ATF4-dependent signaling in mouse liver (Dong, K et al., 2015). FGF21 also shows anti-inflammatory properties in multiple tissues and plays a crucial role in the balance of the pro-inflammation/anti-inflammation system (Li, F. et al., 2018). Liver is the most sensitive organ in response to metabolic disorders and the inflammatory situation (Luo et al., 2017). For example, Fgf21 knocking-out, as depicted in Fgf21-null mice, produces more inflammation in mouse liver; whereas administration of FGF21 analogs, such as LY2405319, improve hepatic steatosis, reduce inflammation, promote mitochondrial functions, and reverse liver fibrosis (Tanaka et al, 2015; Lee et al., 2016). In addition, Fgf21-null mice showed more oxidative stress and inflammation in acetaminophen
Nuclear factor erythroid 2-related factor 2 (Nrf2) is another transcription factor that regulates the expression of cytoprotective genes against oxidative damage and inflammation (Bellezza et al., 2018). Actions of FGF21 and Nrf2 seem to be mutual: for instance, APAP-induced hepatic Nrf2 expression was impaired in Fgf21-null mice while ethanol-induced FGF21 expression was attenuated in Nrf2-null mice (Ye, D et al., 2014; Chen et al., 2017). In addition, administration of FGF21 prevents diabetic renal injury by reducing renal lipid accumulation, inflammation and oxidative stress; as well as prevent hyperglycemia-induced fibrogenesis in human renal mesangial cells through inhibiting STAT5 signaling pathway (Zhang et al., 2013; Li et al., 2017). Pre-treatment with FGF21 also ameliorates phenylephrine (PE)-induced cardiac hypertrophy in mice and reduces inflammation by decreasing Nuclear factor kappa B (NF-kB) activity (Planavila et al., 2013). In the pancreas, FGF21 acts in an autocrine/paracrine manner (Coate et al., 2017; Kliwer et al., 2019), and appears to alleviate acute pancreatic injury in experimental pancreatitis via activation of the Sirt1-autophagy signaling pathway (Chen et al., 2020).

1.4 Cisplatin (CDDP)

Cisplatin (CAS No. 15663-27-1, cis-diamminedichloroplatinum (II), CDDP) is a platinum (II) coordination compound with a cis square planar geometry. Since it was introduced for its anti-cancer effects in 1965 and firstly approved by FDA for cancer
treatment in 1978, there was an increasing interest in coordination complexes of platinum, palladium, and other noble metals in cancer treatment (Rosenberg et al., 1965; Kelland, 2007; Frezza et al., 2010). However, after evaluating 13 of platinum-containing analogues in clinical trials, only carboplatin has been approved worldwide with definite advantage over CDDP (Weiss et al., 1993). Nowadays, CDDP is still being used as the front-line chemotherapy that administered intravenously for treatment of numerous human cancers, including lung, ovarian, testicular, bladder, head and neck cancers (Dasari et al., 2014; Aldossary et al., 2019). In the cytoplasm, CDDP becomes activated once the chloride atoms are displaced by water molecules. Its pharmacological modes of action are associated with its ability to 1) crosslink with the purine bases on the DNA, 2) interfere with DNA repair mechanisms, and 3) induce apoptosis in cancer cells (Dasari et al., 2014). However, Cisplatin therapy is largely restricted by its side effects including nephrotoxicity, hepatotoxicity, ototoxicity and cardiotoxicity (Madias and Harrington, 1978). Among these, hepatotoxicity occurs when cisplatin is administered at relatively high doses and/or chronic usage (Cavalli et al., 1978; Cersosimo, 1993; Pollera et al., 1987).

In clinical, CDDP is administered through intravenous route as an infusion. It is administered safely and with acceptable tolerance at doses of 50-120 mg/m² every 3-4 weeks (Brock et al., 1986). In addition, CDDP can be given by 20 mg/m²/day for 5 days every 3 weeks to treat metastatic testicular tumors (Yoshida et al., 2000). It is
preferentially taken up and accumulated in liver and kidney (Stewart et al., 1982). CDDP causes nephro- and hepatotoxicity through triggering signaling cascades in oxidative stress and inflammation. It has been primarily demonstrated that CDDP induced stress accompanied by activated ATF4 signaling or an increase of Nrf2 expression (Omar et al., 2016; Kuo et al., 2016). CDDP induces inflammation in kidney through NF-κB pathway, evidenced by increased expression of tumor necrosis factor (TNF) α, interleukin (IL)-1β and IL-6 (Zhang et al., 2008). In addition, CDDP caused hepatitis (liver inflammation) via NF-κB activation (El-Shitany et al., 2017). Due to the considerable side effects in CDDP treatment, combination therapy of CDDP with other drugs has been applied to treat cancers while reducing toxicities. For example, cimetidine, an OCT2 inhibitor, prevents OCT2-dependent renal uptake of CDDP and thus reduces toxicity of CDDP in kidney (Katsuda, et al., 2010). In addition, several studies reported that tangeretin, ginsenoside Rg1, vitamin E, and maltol, via repressing oxidative stress and inflammation, attenuated CDDP-induced liver and kidney injury (Omar et al., 2016; Gao et al., 2017; Hemati et al., 2012; Mi et al., 2018).

1.5 Activator protein (AP)-1

Activator protein (AP)-1 protein, which are characterized by highly conserved dimeric basic leucine zipper (bZIP) motif, generally as homodimers composed of two Jun family member (c-Jun, JunB, and JunD) or as heterodimers composed of one Jun family
member and one Fos family member (c-Fos, Fra-1, Fra-2, and FosB) providing for a multiplicity of regulatory control (Angel et al., 1991). In the liver, AP-1 is primarily in the form of c-Jun/c-Fos heterodimer, which has a high affinity for binding to a consensus sequence known as the 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element [TRE, 5’-TGA(C/G)TCA-3’] (Angel et al., 1991). AP-1 proteins participate in various cellular processes including cell survival, proliferation, apoptosis, differentiation, and cell migration and transformation, as well as stress response (Chang et al., 2001; Ye, N et al., 2014). AP-1 is commonly known as a pro-inflammatory factor that is mainly activated by upstream JNK signaling and is believed to directly control downstream cytokines, such as TNFα, IL-1 and IL-2 by directly binding to their promoter sequences (Sirum-Connolly et al., 1991; Raingeaud et al., 1995; Karin et al., 1997; Ye, N et al., 2014).

1.6 Aims

The promising effects of FGF21 in regulation of metabolic homeostasis and protection against stress and injury has prompted an interest in characterizing the molecular mechanisms of its regulation. The study objective of my dissertation focuses on characterization of the effects of AP-1 signaling in CDDP-induced Fgf/FGF21 expression, the impact of Fgf/FGF21 on the progression of CDDP-induced liver injury, and its pathophysiological implications. Specifically, I determined the regulation of Fgf/FGF21 mRNA and protein expression by CDDP and determined the effect of CDDP-
induced AP-1 activation on Fgf/FGF21 expression. In addition, I determined the impact of gain or loss of Fgf/FGF21 function on the progression of CDDP-induced hepatotoxicity.
CHAPTER 2 METHODOLOGY

Chemicals and reagents

Analysis kits of serum alanine aminotransferase (ALT; cat. # A7526-150) and aspartate aminotransferase (AST; cat. # A7561-150) were purchased from Pointe Scientific, Inc. (Ann Arbor, MI). Analysis kits of glucose (cat. # TR15221), triglycerides (cat. # TR22421) and cholesterol (cat. # TR13421) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Total Bile Acid Assay Kit (cat. # STA-631) was purchased from Cell Biolabs Inc. (San Diego, CA). SuperScript II reverse transcriptase (cat. # 18064022), TRIZol reagents (cat. # 15-596-018), SYBR Select Master Mix (cat. # 4472919), Lipofectamine 2000 transfection reagent (cat. # 11668027), NE-PER Nuclear and cytoplasmic extraction reagent kit (cat. # 78833), and mem-PER Plus membrane protein extraction kit (cat. # 89842) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA).

Goat anti-mouse IgG horseradish peroxidase (HRP)-linked (cat. #31430), goat anti-rabbit IgG horseradish peroxidase (HRP)-linked (cat. # 31460), anti-rabbit biotin conjugate (cat. # 31820) and avidin HRP-linked (cat. # 21130) secondary antibodies were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Amyloglucosidase (cat. #A1602), Anti-Actin Antibody (cat. # MAB1501), Chemiluminescent HRP Substrate (cat. # WBKLS0100) for Western blotting and Chromatin immunoprecipitation (ChIP) assay kit (#17-295) were purchased from Millipore Sigma (Billerica, MA). Anti-FGF21
antibody (cat. # ab171941) and Anti-KLB antibody (cat. # ab106794) were purchased from Abcam (Cambridge, MA). Phospho-JNK antibody (cat. # 4668), JNK antibody (cat. # 9252), Phospho-c-Jun antibody (cat. # 3270), c-Jun antibody (cat. # 9165), Phospho-c-Fos antibody (cat. #5348), c-Fos antibody (cat. #2250) and Histone H3 antibody (cat. # 4499) were purchased from Cell Signaling Technology Inc. (Danvers, MA). FGF21 Mouse/Rat ELISA (cat. # RD291108200R) were purchased from BioVendor, LLC (Asheville, NC).

Zymoclean Gel DNA Recovery kit (cat. # D4001), Zymo Plasmid Miniprep kit (cat. # D4036) and DNA Clean & Concentrator kit (cat. # D4013) were purchased from Zymo Research Corporation (Irvine, CA). pGL3-basic vector (cat. # E1751) was purchased from Promega (Fitchburg, WI). All other reagents and chemicals, unless specifically indicated, were purchased from Thermo Fisher Scientific Inc. (Waltham, MA).

Animals and treatment

Approximately eight-week-old adult male C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Breeding pairs of Fgf21-null mice and corresponding wild-type mice were kindly provided by Dr. Steven Kliewer (UT Southwestern Medical Center, Dallas, TX) (Potthoff et al., 2009). The mice were housed according to the guidance of Association for Assessment and Accreditation of Laboratory Animal Care International at St. John’s University animal facility under a standard 12-h
light: dark cycle with free access to regular rodent chow and water ad libitum. Groups of six mice were administered CDDP (3, 10 and 16 mg/kg, i.p., dissolved in saline) once daily for 4 days. Control group received saline. All injections were administered at a volume of 10 ml/kg. Mouse status was monitored daily. Mouse sera were isolated and stored at -80°C until use. Mouse tissues were collected, snap-frozen in liquid nitrogen, and stored at -80°C. One portion of freshly harvested liver was fixed in 10% formalin solution and processed for histological evaluation.

**DEX-CDDP co-treatment study.** Groups of six mice were administered DEX (2 mg/kg; i.p., dissolved in corn oil) once daily for 7 days, followed by CDDP (16 mg/kg; i.p., dissolved in saline) once daily for 4 days. Control group received the corresponding vehicle. Mouse sera were isolated and stored at -80°C until use. Tissues were collected, snap-frozen in liquid nitrogen, and stored at -80°C. One portion of freshly harvested liver was fixed in 10% formalin solution and processed for histological evaluation.

**Histopathology**

Mouse livers were fixed in 10% formalin solution. Liver sections (4 µm in thickness) were then processed for Hematoxylin and Eosin (H&E) staining.

**Quantification of serum levels of triglycerides, total cholesterol, glucose and total bile acids**
Freshly collected whole blood was centrifuged at 5,000 rpm for 10 minutes at 4 °C to allow serum separation. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using Liquid ALT and AST Reagent Sets (Pointe Scientific; Ann Arbor, MI), respectively. Triglycerides, total cholesterol, glucose and total bile acids were measured using respective colorimetric assays kits as mentioned above. All measurements were performed according to the manufacturer’s instructions.

Quantification of triglycerides, total cholesterol, glycogen, and total bile acids in mouse liver

Hepatic lipid contents were extracted as described previously (Tanaka et al., 2008). In brief, livers were homogenized in 1 ml of buffer containing 18 mM Tris, pH 7.5, 300 mM mannitol, 50 mM EGTA, and 0.1 mM phenylmethysulfonyl fluoride. 100 µl of homogenates were mixed with 1 ml of chloroform/methanol (2:1, v/v) and incubated overnight at room temperature with occasional shaking. 200 µl of H2O was then added, vortexed, and centrifuged for 5 min at 3,000 g, and the lower lipid phase was collected and dried by centrifugal concentrators (Labconco corporation, Kansas City, MO). The lipid pellets were then dissolved in a mixture of 90 µl of isopropanol and 10 µl of Triton X-100. Triglycerides and total cholesterol levels were measured using the Triglycerides
and Total Cholesterol Liquid Stable Reagent (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer’s instructions.

Hepatic glycogen was measured after digesting glycogen into glucose, followed by glucose measurement using Glucose Hexokinase Reagents (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer’s instructions. In brief, 10 μl of homogenate samples were mixed with 2 μl of 2.0 M Na Acetate buffer (pH 5.7), containing amyloglucosidase (Millipore sigma, Billerica, MA) at a concentration of 1 unit/sample. Samples were digested at 55° for 45 min and then processed for measurement.

Total bile acid levels in mouse liver were measured using colorimetric assays (Cell Biolabs Inc., San Diego, CA) according to the manufacturer’s instructions.

**Quantification of serum Fgf21 protein level**

Mouse serum samples were processed for quantification of serum Fgf21 levels by using BioVendor’s Rat/Mouse FGF21 ELISA kit (Asheville, NC) per the manufacturer’s instructions.

**Cell culture and treatment**

Mouse Hepa1c1c7 hepatoma cells (cat. # CRL-2026; ATCC®; Manassas, VA) were cultured in Alpha Minimum Essential Medium (cat. # SH30265FS); human HepG2 cells
(cat. # HB-8065; ATCC®; Manassas, VA) and SkHep1 cells (cat. # HTB-52; ATCC®; Manassas, VA) were cultured in Eagle's Minimum Essential Medium (cat. # SH3002401); human SNU423 cells (cat. # CRL-2238; ATCC®; Manassas, VA) and SNU449 cells (cat. # CRL-2234; ATCC®; Manassas, VA) were cultured in RPMI-1640 Medium (cat. # 11-875-093); human Huh7 cells were cultured in Dulbecco's Modified Eagle Medium (cat. # 11-965-092), all the medium was supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2.

Cells were cultured and allowed to grow to 85-90% confluence. Cultured hepatoma cells, in tetraplicate, were treated with 0.1, 0.3 and 1 μM of CDDP for 6, 12, 24 and 48 hours, respectively. Control group receive 0.1% DMSO. Cells were harvested after treatments and total RNAs were extracted.

_CDDP-SR11302 co-treatment studies._ Cultured hepatoma cells, in tetraplicate, were incubated with 30 µM SR11302 for 24 hours, with or without co-treatment of 0.3 µM CDDP for 6 hours. Control group receive 0.1% DMSO. Cells were harvested 24 hours later and total RNAs were extracted.

_TPA-SR11302 co-treatment studies._ Cultured hepatoma cells, in tetraplicate, were incubated with 30 µM SR11302 for 24 hours, with or without co-treatment of 0.1 µM TPA for 6 hours. Control group receive 0.1% DMSO. Cells were harvested 24 hours later and total RNAs were extracted.
CDDP-DEX or CDDP-BNF co-treatment studies. Mouse hepatica 7 and human HepG2 hepatoma cells were incubated with 1 µM DEX or 0.1 µM BNF for 24 hours, and then treated with 1 µM CDDP for 48 hours (n=6/treatment). Control group receive 0.1% DMSO. Cells were then processed for MTT assay.

Total RNA extraction and quantification

Total RNAs were extracted from cultured hepatoma cells or mouse tissues using TRIzol reagents according to the manufacturer's instructions (Thermo Fisher Scientific Inc., Waltham, MA). RNA integrity was assessed by electrophoresis on a denatured agarose gel containing 3-(N-morpholino) propanesulfonic acid (MOPS) and formaldehyde. Total RNA concentrations were quantified at 260nm with Eppendorf Biospectrometer (Hauppauge, NY). RNA samples with an A260/A280 ratio between 1.8-2.0 were used for further analysis.

Quantitative real time-PCR (qRT-PCR) assay

Total RNAs were reversely transcribed into cDNA using SuperScript II reverse transcriptase following the manufacturer’s instructions (Thermo Fisher Scientific Inc., Waltham, MA). Quantitative PCR was performed using SYBR Select Master Mix (Thermo Fisher Scientific Inc., Waltham, MA) in an AriaMx Real-Time PCR system (Agilent Technologies, Santa Clara, CA). Data were calculated according to the
comparative delta-delta CT method and represented as relative fold of the control. The primers used in qRT-PCR analysis were listed in Table 2.1. All of primers were designed with PubMed Primer-BLAST or Primer3 software (NIH) and synthesized by Integrated DNA Technologies (Coralville, IA) or Eurofins Genomics (Louisville, KY).

**Protein extraction and Western blots**

Membrane and cytoplasmic proteins were separated and extracted using Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific Inc., Waltham, MA). Cytoplasmic and nuclear proteins were extracted from cultured hepatoma cells and mouse liver using NE-PER® Nuclear and Cytoplasmic Extraction Reagent kit according to the manufacturer's instructions (Thermo Fisher Scientific Inc., Waltham, MA). The protein concentrations were quantified spectrophotometrically at 280 nm. Equal amounts of protein (60 µg/lane) were resolved on a 15% SDS-polyacrylamide gel. Following electrophoresis, proteins were transferred to a 0.45 µm polyvinyl difluoride (PVDF) membrane. Then, the membrane was blocked for 2 hours in 5% BSA in Tris-buffered saline (TBS). The membrane was next incubated overnight with antibodies against Fgf21, Klb, phospho-JNK, JNK, phospho-cJun, c-Jun, phospho-cFos or c-Fos at 4°C. Blotting of β-actin or Histone H3 protein was used as a loading control. After thorough washing, the membrane was incubated with corresponding secondary antibody (1:5,000 in 2.5% BSA in TBS) for 2 h at room temperature. Immunoreactive bands in the membrane were
detected with Immobilon Chemiluminescence reagent (Millipore sigma, Billerica, MA) and ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Hercules, CA). The intensity of protein bands was quantified with ImageJ software (NIH).

**MTT assay for cell viability**

Cultured mouse Hepa1c1c7 and human HepG2 hepatoma cells were seeded in 96-well plates and incubated overnight for cell attachment. After treatment, 20μL of the 4 mg/ml MTT solution was added to each well and incubated at 37°C for 3 hours. The solution was then carefully removed and 100μL DMSO was added to each well for crystal dissolution. Absorbance of the purple color can be spectrophotometrically measured at 570nm with Infinite® 200 PRO micro plate reader (Tecan Inc., Morrisville, NC).

**Seahorse extracellular flux analysis of mitochondrial respiration**

Mitochondrial respiration after CDDP treatment in mouse Hepa1c1c7 and human HepG2 hepatoma cells was analyzed by Agilent Seahorse XF Cell Mito Stress Test using Agilent Seahorse XFp Analyzer (Agilent Seahorse Bioscience, Santa Clara, CA). Oxygen consumption rate (OCR) was measured in function of time and added respiration modulators according to the manufacturer’s instructions. Briefly, the cells were seeded in a Seahorse 6-well XFp Cell Culture microplate the day before the treatment. In addition,
the Seahorse XF Sensor Cartridge was hydrated the day before the assay by filling each well of the XFp Utility Plate with Seahorse XF Calibrant Solution and incubated in a non-CO₂ 37°C incubator for overnight to remove CO₂. After CDDP treatment, the cells were washed three times with assay medium and then incubated in assay medium in a non-CO₂ 37°C incubator for 1 hour. During the assay, mitochondrial function of the cells was analyzed by sequential injections of modulators: oligomycin (1 μM), carbonyl-cyanide-4-(trifluoromethoxy) phenyhydrazone (FCCP, 0.25 μM), a mix of rotenone/antimycin A (0.5 μM).

**Engineering of mouse/human Fgf/FGF21 promoter into pGL3 luciferase reporter vector**

Mouse genomic DNA was used as the template to clone a 2.5-kb fragment containing the 5′-flanking region of mouse Fgf21 gene by PCR (Forward primer, 5′-ATA CTC ATC CCT GGG CGT CT -3′; Reverse primer, 5′-CCA TTC CAT CAG ATC TGC GCT -3′). A human BAC clone (RP11-165B22; obtained from Children’s Hospital Oakland Research Institute, Oakland, CA) was used as the template to clone a 2.7-kb fragment containing the 5′-flanking region of human FGF21 gene by PCR (Forward primer, 5′-CAA GCT AGC GCA TTT TCT CTG GTC CAA GGC -3′; Reverse primer, 5′-CAA AGA TCT CTC TTT CGG CTG GAT CCT CA -3′). PCR products were gel-purified and engineered into the pGL3-basic vector (cat. # E1751; Promega; Fitchburg,
The cloned recombinant pGL3 luciferase reporter constructs were validated by DNA sequencing (Eurofins Genomics; Huntsville, AL).

**Partial deletion assay of Fgf/FGF21 gene promoter**

Partial truncations of mouse and human Fgf/FGF21 gene promoter were achieved via PCR, similarly as previously described (Bu et al., 2017). The locations and sequences of the forward primers, and the reverse primers were listed in Table 2.2. The sequences of partially truncated Fgf/FGF21 promoters that were engineered into empty pGL3-basic vector (cat. # E1751; Promega; Fitchburg, WI) were validated by DNA sequencing (Eurofins Genomics; Huntsville, AL).

**Transient Transfection and Luciferase Reporter Assay**

Recombinant pGL3 reporter constructs were transfected into mouse Hepa1c1c7 or human HepG2 cells, using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions. Briefly, the cells were seeded onto 96-well tissue culture plates one day prior to transfection. Mouse/human pGL3-Fgf/FGF21 (180 ng) or its empty pGL3-basic vector (cat. # E1751; Promega; Fitchburg, WI), plus phRL-CMV vector encoding Renilla luciferase (10 ng) were mixed with Lipofectamine 2000 with 1:1 (v/v) ratio and then dispensed to each well. After transfection for 24 hours, the medium was replaced with fresh medium with
or without CDDP treatment at indicated concentrations. 24 hours later, the cells were lysed and processed for Dual-Luciferase® reporter assays per the manufacturer's instructions (cat. # E1910; Promega; Fitchburg, WI).

**Chromatin Immunoprecipitation (ChIP)-qPCR assays**

The putative AP-1 binding sites were further analyzed in mouse Hepa1c1c7 and human HepG2 cells by ChIP-qPCR assays with anti-c-Jun antibody using EZ-ChIP assay kit (cat. # 17-371RF; Millipore; Billerica, MA) according to the manufacturer's instructions. Briefly, cells were cultured in T-75 flask and treated with CDDP (0.3 µM) for 6 hours before cross-linking with formaldehyde at a final concentration of 1% for 10 min at room temperature. Glycine was added to final concentration of 0.125 M to terminate the cross-linking reaction. 10 µg of anti-c-Jun, 1 µg IgG (negative control), and 1 µg RNA Pol II (positive control) antibody were used for immunoprecipitations. Precipitated DNAs were purified and dissolved in 20 µl of water. 4 µl of DNA solution was used for the quantitative real-time PCR reaction. Quantitative PCR was performed using SYBR Select Master Mix (Thermo Fisher Scientific Inc., Waltham, MA) in an AriaMx Real-Time PCR system (Agilent Technologies, Santa Clara, CA). The primers flanking the specific AP-1 binding sites are given in Table 2.3.

**Statistical analysis**
Data are expressed as Mean ± Standard Error of the Mean. Data of three or more groups were analyzed by one-way analysis of variance, followed by Duncan's post-hoc test using Sigmaplot (Systat Software, Inc., CA). Data of two-treatment groups were analyzed by Student’s T-test. Statistical significance was considered when $p < 0.05$. 
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Table 2.2 DNA sequences of PCR cloning primers of mouse and human Fgf/FGF21 gene promoter.

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<th>mFgf21/hFGF21 promoter</th>
<th>Promoter region</th>
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Table 2.3 DNA sequences of qRT-PCR primers flanking AP-1 binding sites in mouse and human Fgf/FGF21 gene promoter for the ChIP-qPCR assays

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<td>CTGAAACAGTTTGTCCTCAGT</td>
<td>AGCCTGGACTCTAAGTC</td>
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CHAPTER 3 RESULTS

3.1 Regulation of Mouse and Human Fgf/FGF21 expression by CDDP

3.1.1 Dose-dependent up-regulation of Fgf21 expression in mice by CDDP

I first conducted dose-response study of Fgf21 mRNA expression by CDDP in mouse liver. As shown in Fig. 3.1, 3, 10 and 16 mg/kg of CDDP induced Fgf21 mRNA expression by 2.2-, 6.2- and 11.9-fold in mouse liver, respectively.

In my remaining dissertation research, two doses of CDDP treatment (3 and 16 mg/kg) were selected to characterize the regulation of Fgf21 mRNA and protein expression by CDDP in mice.

3 and 16 mg/kg of CDDP increased Fgf21 protein level in mouse livers by 2.2- and 2.4-fold, respectively (Fig. 3.2).

As liver-derived Fgf21 can function as an endocrine hormone, we next determined the alteration of serum levels of Fgf21 protein following CDDP treatment. Baseline serum Fgf21 protein level is around 650 pg/ml, as shown in the control group. After four-day once daily treatment, 3 and 16 mg/kg of CDDP increased serum Fgf21 protein levels to 1,440 pg/ml and 2,900 pg/ml, respectively (Fig. 3.3).

In addition to liver, Fgf21 is also expressed in other mouse tissues such as kidney and heart. As shown in Fig. 3.4A, 3 and 16 mg/kg of CDDP increased Fgf21 mRNA expression by 3- and 19-fold in mouse kidneys, respectively. In mouse heart, 3 and 16
mg/kg of CDDP increased Fgf21 mRNA expression by 2- and 2.5-fold, respectively (Fig. 3.4B).

**Figure 3.1 Dose-dependent regulation of Fgf21 mRNA expression by CDDP in mouse livers.**

Adult male C57BL/6 mice were given i.p. administration of CDDP at doses of 3, 10 and 16 mg/kg once daily for 4 days (n=6/treatment). Control received saline. On day 5, mouse livers were collected and processed for mRNA analysis by RT-PCR. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between control and CDDP treatment.
Figure 3.2 Representative immunoblotting images and quantitative presentation of Fgf21 protein after CDDP treatment in mouse livers.

Adult male C57BL/6 mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment). Control received saline. On Day 5, mouse livers were collected and processed for Fgf21 protein analysis by Western blots. β-actin was used as the loading control. The data are presented as means ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between control and CDDP treatment.
**Figure 3.3 Regulation of serum Fgf21 protein levels by CDDP in mice.**

Adult male C57BL/6 mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment). Control received saline. On Day 5, mouse sera were isolated for Fgf21 protein measurement by ELISA. The data are presented as means ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and CDDP treatment.
Figure 3.4 Regulation of Fgf21 mRNA by CDDP in mouse kidney (A) and heart (B).

Adult male C57BL/6 mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment). Control received saline. On day 5, mouse kidney and heart were collected and processed for mRNA analysis by RT-PCR. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between control and CDDP treatment.
3.1.2 Time- and concentration-dependent regulation of Fgf/FGF21 expression in cultured mouse and human hepatoma cells by CDDP

To determine whether CDDP similarly regulates mouse and human Fgf/FGF21 expression, mouse Hepa1c1c7 and human HepG2 hepatoma cells were used and treated.

In mouse Hepa1c1c7 hepatoma cells, CDDP up-regulated Fgf21 mRNA expression in both time- and concentration-dependent manners. 0.1, 0.3 and 1 μM of CDDP increased Fgf21 mRNA expression by 3.1-, 10.7- and 7.1-fold after 6 hours, respectively (Fig. 3.5A). 0.1, 0.3 and 1 μM of CDDP increased Fgf21 mRNA expression by 6.4-, 6.4- and 6-fold after 12 hours, respectively (Fig. 3.5A). In human HepG2 hepatoma cells, 0.1, 0.3 and 1 μM of CDDP increased FGF21 mRNA expression by 4.7-, 5- and 8.7-fold after 6 hours, respectively (Fig. 3.5B). 0.1, 0.3 and 1 μM of CDDP increased FGF21 mRNA expression by 1.8-, 4.6- and 6.3-fold after 12 hours, respectively (Fig. 3.5B). However, after 24 hours, CDDP, at any concentrations, did not induce mouse or human Fgf/FGF21 mRNA expression (Fig. 3.5).

Because protein translation occurs after DNA is transcribed into mRNA, we investigated Fgf/FGF21 protein expression by CDDP after 24 hours. 0.1, 0.3 and 1 μM of CDDP increased Fgf21 protein by 1.4-, 2.1-, and 1.8-fold, respectively in mouse Hepa1c1c7 hepatoma cells (Fig. 3.6A). In human HepG2 hepatoma cells, 0.3 and 1 μM of CDDP increased FGF21 protein levels by more than 1.5-fold (Fig. 3.6B).
Figure 3.5 Concentration- and time-dependent regulation of Fgf/FGF21 mRNA expression by CDDP in cultured mouse and human hepatoma cells.
Mouse Hepa1c1c7 (A) and human HepG2 (B) hepatoma cells were treated with CDDP (0.1, 0.3 and 1 μM) for 6, 12, and 24 hours, respectively. Control received 0.1% DMSO.

Cells were then harvested and processed for mRNA analysis by RT-PCR. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and CDDP treatment.
Figure 3.6 Representative immunoblotting images and quantitative presentation of Fgf/FGF21 protein in cultured mouse and human hepatoma cells after CDDP treatment.

Mouse Hepa1c1c7 (A) and human HepG2 (B) hepatoma cells were treated with CDDP (0.1, 0.3 and 1 μM) for 24 hours. Control received 0.1% DMSO. Cells were then harvested and processed for Fgf/FGF21 protein analysis by Western blots. β-actin was used as the loading control. The data are presented as means ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between control and CDDP treatment.
3.1.3 Regulation of Klb and Fgfr expression by CDDP in wild-type and Fgf21-null mouse livers

FGF21 action through FGFR1 requires β-klotho (KLB) (Degirolamo et al., 2016). KLB is predominantly expressed in the liver in both mouse and human (Zou et al., 2018), and participates in lipid, glucose, and energy metabolism (Ogawa et al., 2007). We determined regulation of Klb mRNA and protein expression by CDDP in mouse liver. After four-day daily treatment, 3 and 16 mg/kg of CDDP increased Klb mRNA expression by 2.5- and 9.7-fold in mouse livers, respectively (Fig. 3.7A). CDDP (16 mg/kg) increased Klb membrane protein levels by 1.7-fold in wild-type mouse livers (Fig. 3.7B). In contrast, in Fgf21-null mouse livers, the induction of Klb mRNA and protein expression by CDDP is abolished (Fig. 3.7).

In addition, it has been reported that all of FGFR1-4 can mediate FGF21 signaling. Amongst them, FGFR1 is the key receptor for FGF21 action (Lin et al., 2017; Kurosu et al., 2007; Yie et al., 2012). As shown in Fig. 3.8A, 3 and 16 mg/kg of CDDP increased Fgfr1 mRNA expression in wild-type mouse livers by 3- and 2.7-fold, respectively, but not in Fgf21-null mouse livers. In contrast, 16 mg/kg of CDDP tended to decrease Fgfr2, Fgfr3 and Fgfr4 expression in both wild-type and Fgf21-null mouse livers (Fig. 3.8B, C and D). Disruption of Fgf21 function as observed in Fgf21-null mice, did not apparently alter mRNA expression of Fgfrs (Fig. 3.8).
Figure 3.7 Regulation of Klb by CDDP in wild-type and Fgf21-null mouse livers.

A, Regulation of Klb mRNA expression by CDDP; B, Representative immunoblotting images and quantitative presentation of Klb membrane protein in wild-type and Fgf21-null mouse livers after CDDP treatment. Adult male wild-type and age-matched male Fgf21-null mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment/genotype). Control received saline. On Day 5, mouse livers were collected and processed for mRNA analysis by RT-PCR or membrane protein analysis by Western blots. 18S and β-actin were used as the loading control, respectively. The data are presented as means ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between control and CDDP treatment. Single daggers (†) represent a statistical difference (p < 0.05) between wild-type and Fgf21-null mice.
Figure 3.8 Regulation of Fgfr1 (A), Fgfr2 (B), Fgfr3 (C) and Fgfr4 (D) mRNA by CDDP in wild-type and Fgf21-null mouse livers.

Adult male wild-type and age-matched male Fgf21-null mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment/genotype). Control received saline. On day 5, mouse livers were collected.
and processed for mRNA analysis by RT-PCR. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and CDDP treatment. Single daggers (†) represent a statistical difference ($p < 0.05$) between wild-type and Fgf21-null mice.
3.1.4 Regulation of Pgc1α and Gdf15 by CDDP in wild-type and Fgf21-null mouse livers

In the liver, PGC1α is a known downstream target gene of FGF21 signaling (Cariello et al., 2014). We next determined the expression of Pgc1α by CDDP in mouse liver. After four-day once daily treatment, 3 and 16 mg/kg of CDDP increased Pgc1α mRNA expression in wild-type mouse livers by 3- and 13.6-fold, respectively (Fig. 3.9A). Whereas in Fgf21-null mouse lives, 3 and 16 mg/kg of CDDP increased Pgc1α mRNA expression by 3.5- and 4-fold, respectively (Fig. 3.9A).

Similar to FGF21, growth differentiation factor (GDF) 15 is another metabolic stress-induced cytokine, which can be specifically induced by mitochondrial stress (Khan et al., 2017). We also determined the regulation of Gdf15 mRNA expression by CDDP in wild-type and Fgf21-null mouse liver. As shown in Fig 3.9B, 16 mg/kg of CDDP increased Gdf15 mRNA expression in wild-type mouse livers by 4.2-fold, but not in Fgf21-null mouse lives.
Figure 3.9 Regulation of Pgc1α (A) and Gdf15 (B) mRNA expression by CDDP in wild-type and Fgf21-null mouse livers.

Adult male wild-type and age-matched male Fgf21-null mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment/genotype). Control received saline. On day 5, mouse livers were collected and processed for mRNA analysis by RT-PCR. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between control and CDDP treatment. Single daggers (†) represent a statistical difference (p < 0.05) between wild-type and Fgf21-null mice.
3.2 CDDP induced Fgf/FGF21 expression through AP-1 activation

3.2.1 CDDP did not activate ATF4 or Nrf2 signaling in mouse liver

ATF4 and Nrf2 are two stress-responsive transcription factors (Rössler et al., 2017). Endoplasmic reticulum (ER) stress can induce CHOP (C/EBP homologous protein) expression via eIF2α-ATF4 signaling. My results showed that CDDP decreased ATF4 nuclear protein, phospho-eIF2α protein levels and the downstream CHOP mRNA expression (Fig. 3.10A, B, C and D). In addition, CDDP did not apparently increase mRNA or nuclear protein level of Nrf2, as well as mRNA expression of Nqo (NAD(P)H dehydrogenase) 1 or Ho (heme oxygenase) 1, two downstream target genes of Nrf2 signaling (Fig. 3.10 E, F, G and H).
Figure 3.10 Regulation of Atf4 and Nrf2 signaling by CDDP in mouse livers.

A, Regulation of Atf4 mRNA expression; B and C, Representative immunoblotting image and quantitative presentation of Atf4 and phospho-elf2α protein levels; D, Regulation of CHOP mRNA expression; E, Regulation of Nrf2 mRNA expression; F, Representative immunoblotting image and quantitative presentation of Nrf2 protein levels; G and H, Regulation of Nrf2, Nqo1 and Ho1 mRNA expression. Adult male C57BL/6 mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment). Control received saline. On day 5, mouse livers were collected and processed for mRNA analysis by RT-PCR, or protein analysis by Western blots. β-actin and Histone H3 were used as the loading control for cytoplasmic and
nuclear protein, respectively. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and CDDP treatment.
3.2.2 CDDP produced mitochondrial stress in cultured mouse and human hepatoma cells

The effect of CDDP on the mitochondrial respiration were determined by performing Seahorse XF Cell Mito Stress Tests. The impacts of CDDP on the oxygen consumption rate (OCR) levels are shown in Fig. 3.11. CDDP produced mitochondrial stress in both mouse and human hepatoma cells, indicated by a decrease in basal respiration, in ATP production, and especially in maximal respiration (Fig. 3.11).
Figure 3.11 Effects of CDDP on mitochondrial respiration in mouse and human hepatoma cells.

A, mouse Hepa1c1c7 cells treated with 0.3 μM CDDP; B, mouse Hepa1c1c7 cells treated with 1 μM CDDP; C, human HepG2 cells treated with 0.3 μM CDDP; and D, human HepG2 cells treated with 1 μM CDDP for 24 hours. Control received 0.1% DMSO. Cells
were harvested and processed for Seahorse XF Cell Mito assay. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and CDDP treatment.
3.2.3 Regulation of JNK-cJun-AP1 signaling by CDDP in mouse livers, as well as cultured mouse and human hepatoma cells

AP-1 is primarily in the form of c-Jun/c-Fos heterodimer in the liver. 16 mg/kg of CDDP induced c-Jun and c-Fos mRNA expression in mouse livers by 6.3- and 5.6-fold, respectively (Fig. 3.12A and B).

c-Jun N-terminal Kinases (JNK) is a common upstream molecule of cJun-AP1 signaling. 16 mg/kg of CDDP increased phospho-JNK and phospho-c-Jun nuclear protein expression in mouse livers (Fig. 3.12D and E). However, CDDP did not alter phospho-c-Fos nuclear protein expression in mouse livers (Fig. 3.12F).

In mouse Hepa1c1c7 and human HepG2 hepatoma cells, CDDP concentration-dependently induced c-Jun/JUN mRNA and nuclear protein expression. In mouse Hepa1c1c7 cells, 0.3 and 1 μM CDDP increased c-Jun mRNA expression by 2.1-, and 1.9-fold, respectively (Fig. 3.13A); while 0.3 and 1 μM CDDP increased c-Jun nuclear protein level by 1.6-, and 1.4-fold, respectively (Fig. 3.13B). In human HepG2 cells, 0.3 and 1 μM CDDP increased c-JUN mRNA expression by 5.1- and 7-fold, respectively (Fig. 3.13C); while 0.3 and 1 μM CDDP increased c-JUN nuclear protein level by 1.4-, and 1.5-fold, respectively (Fig. 3.13D).
Figure 3.12 Regulation of AP-1 expression by CDDP in mouse livers.

A and B, Regulation of c-Jun and c-Fos mRNA expression; C, Representative immunoblotting image; D, E and F, quantitative presentation of phospho-JNK, phospho-c-Jun and phospho-c-Fos nuclear protein levels. Adult male C57BL/6 mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment). Control received saline. On day 5, mouse livers were collected and processed for mRNA analysis by RT-PCR, or protein analysis by Western blots. Total protein antibodies were used as the loading control for corresponding phosphorylated
protein. The data are presented as mean ± SEM. Asterisks (*) represent a statistical
difference ($p < 0.05$) between control and CDDP treatment.
Figure 3.13 Regulation of c-Jun/JUN mRNA and nuclear protein levels by CDDP in cultured mouse and human hepatoma cells.
A, Regulation of c-Jun mRNA expression in mouse Hepa1c1c7 hepatoma cells; B, Representative immunoblotting image and quantitative presentation of phospho-c-Jun nuclear protein levels in mouse Hepa1c1c7 hepatoma cells; C, Regulation of c-JUN mRNA expression in human HepG2 hepatoma cells; D, Representative immunoblotting image and quantitative presentation of phospho-c-JUN nuclear protein levels in human HepG2 hepatoma cells. Cells were treated with CDDP (0.1, 0.3 and 1 μM) for 6 or 24 hours. Control received 0.1% DMSO. Cells were harvested and processed for mRNA analysis by RT-PCR after 6 hours or protein analysis by Western blots after 24 hours. Total protein antibodies were used as the loading control for corresponding phosphorylated protein. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference \( (p < 0.05) \) between control and CDDP treatment.
3.2.4 Regulation of Fgf/FGF21 mRNA expression by SR11302-CDDP cotreatment in cultured mouse and human hepatoma cells

SR11302 is a selective AP-1 inhibitor (Ye et al., 2014). SR11302 pre-treatment attenuated CDDP-induced Fgf/FGF21 mRNA expression in both mouse Hepa1c1c7 and human HepG2 cells, indicating important roles of AP-1 signaling in CDDP-induced Fgf/FGF21 expression (Fig. 3.14).
Figure 3.14 Effect of SR11302 cotreatment on CDDP-induced Fgf/FGF21 mRNA expression in cultured mouse and human hepatoma cells.

Mouse Hepa1c1c7 (A) and human HepG2 (B) hepatoma cells were treated with CDDP (0.3 µM) in the presence or absence of SR11302 (30 µM) pretreatment. Control received 0.1% DMSO. Cells were harvested and processed for Fgf/FGF21 mRNA analysis by RT-PCR. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between control and treatment. Single daggers (†) represent a statistical difference (p < 0.05) between cotreatment group (SR11302+CDDP) vs. CDDP-only group.
3.2.5 Luciferase reporter assays of mouse or human Fgf/FGF21 promoters following CDDP treatment

*In silico* DNA analysis of 2.5-kb of mouse and human Fgf/FGF21 gene promoters (Alibaba 2.1, TRANSFAC® Public) identified several putative AP-1 binding sites [mouse: -397, -431, -465, -499 and -1,347bp upstream of transcription start site (TSS); human: -704, -1,100 and -1,140bp upstream of TSS].

To determine the role of AP-1 signaling in CDDP-induced Fgf/FGF21 expression, we engineered luciferase reporter constructs containing a series of Fgf/FGF21 gene promoters with different length, as described in Fig. 3.15A and B.

In mouse Hepa1c1c7 cells, luciferase activity driven by the 2.3-kb mouse Fgf21 promoter was induced 1.8-fold following 0.3 μM of CDDP treatment (Fig. 3.15C). The truncated mouse Fgf21 promoter that does not contain distal AP-binding site upstream of TSS (-1,347 to -1,340) cannot be activated by CDDP already (Fig. 3.15C).

In human HepG2 cells, similarly, luciferase activity driven by the 2.0-kb human FGF21 promoter was induced 1.7-fold following 0.3 μM of CDDP treatment (Fig. 3.15D). Human FGF21 promoter activity gradually decreased after truncated and cannot be activated by CDDP after removing AP-binding site located at -704 to -697 upstream of TSS (Fig. 3.15D).
These results indicated that the putative AP-1 binding sites within 2.5-kb of mouse and human Fgf/FGF21 promoter are essential for CDDP to induce Fgf/FGF21 expression.
Figure 3.15 Regulation of luciferase reporter activity driven by a series of mouse or human Fgf/FGF21 promoters of different length following CDDP in cultured hepatoma cells.
A and B, schematic representation of partially deleted promoter regions of mouse and human Fgf/FGF21 promoter, respectively. Putative AP-1 binding sites are indicated in the Fgf/FGF21 promoter DNA sequences. A series of Fgf/FGF21 promoters with different length were engineered into pGL3 luciferase reporter vector. C, Mouse Hepa1c1c7 cells were transfected with 2.3-, 1.0- or 0.3-kb of pGL3-Fgf21 reporter construct; D, Human HepG2 cells were transfected with 2.0-, 0.7-or 0.2-kb pGL3-FGF21 reporter construct. Cells were then treated with 0.3 μM CDDP for 24 hours. Control received 0.1% DMSO. Cells were harvested and processed for Dual luciferase reporter assays (Promega; Fitchburg, WI). The data are presented as means ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and CDDP treatment of the same construct. Single daggers (†) represent a statistical difference ($p < 0.05$) between specifically truncated construct and 2.3-kb Fgf21 or 2.0-kb FGF21 gene promoter.
3.2.6 Effects of CDDP on phospho-c-Jun protein binding to mouse and human Fgf/FGF21 gene promoter analyzed by ChIP-qPCR assays

To further determine whether CDDP alter the actual binding of phospho-c-Jun protein to mouse and human Fgf/FGF21 gene promoter, ChIP-qPCR assays were performed.

In mouse Hepa1c1c7 cells, CDDP increased the binding of phospho-c-Jun protein to the DNA fragment located around -1,347bp of mouse Fgf21 gene promoter by 1.7-fold, but not to DNA fragments located around -499bp of mouse Fgf21 gene promoter (Fig. 3.16C). In human HepG2 cells, CDDP increased the binding of phospho-c-JUN protein to the DNA fragments located around -1,140bp of human FGF21 gene promoter by 2.6- and 2-fold, but not apparently to DNA fragments located around -704bp of human FGF21 gene promoter (Fig. 3.16D).
Figure 3.16 CDDP increased the binding of phospho-c-Jun protein to mouse or human Fgf/FGF21 gene promoter.

A and B, schematically showing the locations of ChIP-qPCR primers encompassing the putative AP-1 binding site present in mouse and human Fgf/FGF21 promoter, respectively. C, Mouse Hepa1c1c7 cells after CDDP treatment (0.3 μM, 6 hours) were processed for ChIP-qPCR assays to determine the binding of phospho-c-Jun protein to the putative AP-1 binding site located -1,347bp and -499bp upstream of TSS in mouse Fgf21 gene promoter; D, Human HepG2 cells after CDDP treatment (0.3 μM, 6 hours) were processed for ChIP-qPCR assays to determine the binding of phospho-c-JUN
protein to the putative AP-1 binding site located -1,140bp and -704bp upstream of TSS in human FGF21 gene promoter. Control received 0.1% DMSO. Cells were harvested and processed for ChIP-qPCR assays. Normal IgG was used as the negative control to normalized the phospho-c-Jun binding intensity. Asterisks (*) represent a statistical difference ($p < 0.05$) of phospho-c-Jun binding between control and CDDP treatment.
3.2.7 Regulation of FGF21 and c-JUN mRNA expression by CDDP in other human hepatoma cells

JNK-cJun-AP1 signaling is characterized by induced c-Jun expression. To further evaluate the effects of JNK-cJun-AP1 activation in CDDP-induced FGF21 expression, we determined the regulation of FGF21 and c-JUN expression by CDDP in several other human hepatoma cells.

As shown in Fig. 3.17, in human SNU423 cells, 0.1, 0.3 and 1 μM CDDP induced FGF21 mRNA expression more than 3-fold, and also increased c-JUN mRNA expression by more than 2-fold (Fig. 3.17A and B). In human Skhep1 cells, 1 μM of CDDP induced mRNA expression of FGF21 and c-JUN by 2.2- and 1.6-fold, respectively (Fig. 3.17C and D).

In human SNU449 and Huh7 cell, CDDP at any of selected concentrations (0.1, 0.3 or 1 μM) did not induce FGF21 mRNA expression (Fig. 3.17E and G). In addition, CDDP at any of selected concentrations did not either induce c-JUN mRNA expression in these two cell lines (Fig. 3.17 F and H). These results demonstrated that induction of c-JUN is necessary for CDDP to induce FGF21 expression in human hepatoma cells. Without c-JUN induction, there is no FGF21 induction by CDDP.
Figure 3.17 Regulation of FGF21 (A, C, E and G) and c-JUN (B, D, F and H) mRNA expression by CDDP in cultured human hepatoma cells.

Human SNU423 (A and B), SkHep1 (C and D), SNU449 (E and F) and Huh7 (G and H) hepatoma cells were treated with 0.1, 0.3 and 1 μM of CDDP for 6 hours. Control received 0.1% DMSO. Cells were harvested and processed for mRNA analysis by RT-PCR. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and CDDP treatment.
3.2.8 Regulation of Fgf/FGF21 and c-Jun/JUN mRNA expression by SR11302-TPA cotreatment in cultured mouse and human hepatoma cells

AP-1 is known to be inducible by phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (Li et al., 1999). I next determined whether TPA can similarly induce Fgf/FGF21 expression; and if so whether SR11302 cotreatment can attenuate TPA-induced Fgf/FGF21 expression. TPA induced Fgf21 and c-Jun mRNA expression by 5- and 2.5-fold in cultured mouse Hepa1c1c7 cells, respectively (Fig. 3.18A and B); while TPA induced FGF21 and c-JUN mRNA expression by 5.5- and 6-fold in cultured human HepG2 cells, respectively (Fig. 3.18C and D). SR11302 pre-treatment attenuated TPA-induced Fgf/FGF21 and c-Jun/JUN mRNA expression in both mouse Hepa1c1c7 and human HepG2 cells (Fig. 3.18). These results further indicated the correlation between induction of Fgf/FGF21 and c-Jun/JUN.
Figure 3.18 Effect of SR11302 co-treatment on TPA-induced Fgf/FGF21 and c-Jun/JUN mRNA expression in cultured mouse and human hepatoma cells.

Mouse Hepa1c1c7 (A and B) and human HepG2 (C and D) hepatoma cells were treated with TPA (0.1 µM) in the presence or absence of SR11302 (30 µM) pretreatment.
Control received 0.1% DMSO. Cells were harvested and processed mRNA analysis by RT-PCR. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference \( (p < 0.05) \) between control and treatment. Single daggers (†) represent a statistical difference \( (p < 0.05) \) between cotreatment group (SR11302+TPA) vs. TPA-only group.
3.3 Fgf/FGF21 induction attenuated CDDP-induced hepatotoxicity

3.3.1 CDDP caused body wasting and liver injury in wild-type and Fgf21-null mice

Mouse body weight change following CDDP treatment was monitored daily and shown in Fig. 3.19A. 3 mg/kg of CDDP treatment tended to decrease body weight in both wild-type and Fgf21-null mice. After 4-day treatment, 16 mg/kg of CDDP once daily decreased body weight by 5.2 g and 6 g in wild-type and Fgf21-null mice, respectively (Fig. 3.19A). In addition, CDDP decreased relative liver weight (ratio of liver to body weight) (Fig. 3.19B).

CDDP produced apparent liver injury and inflammation in wild-type mouse livers, evidenced by cell hypertrophy, dilatation and congestion in sinusoids (Fig. 3.20A, B and C). In addition, 16 mg/kg of CDDP increased number of infiltrated immune cells (arrows), and cellular degeneration (circles) (Fig. 3.20C). Disruption of Fgf21 function, as shown in Fgf21-null mice, increased number of infiltrated immune cells (arrows) (Fig. 3.20D). In addition, CDDP increased number of infiltrated immune cells (arrows) and cytoplasmic vacuolation (arrowheads), as well as nuclear pyknosis and cellular degeneration (circles) in Fgf21- null mouse livers (Fig. 3.20E and F).

Moreover, CDDP, especially at the dose of 16 mg/kg, increased serum ALT and AST levels (Fig. 3.21). 16 mg/kg of CDDP increased serum ALT and AST levels even higher in Fgf21-null mice (Fig. 3.21).
We further characterized CDDP-induced inflammation by determining the mRNA expression of several inflammation-related genes (Tnfα, Il-1β, Il-6, and Il-10). CDDP did not apparently alter Tnfα mRNA expression in mouse livers (Fig. 3.22A) but induced mRNA expression of Il-1β, Il-6, and Il-10 in both wild-type and Fgf21-null mouse livers (Fig. 3.22B, C and D). 16 mg/kg of CDDP induced Il-1β, Il-6, and Il-10 by 2.7-, 3.8- and 3.4-fold in wild-type mouse livers, respectively, whereas induced by 2.5-, 4.1-, and 2.5-fold in Fgf21-null mouse livers, respectively (Fig. 3.22B, C and D). In addition, disruption of Fgf21 function, as observed in Fgf21-null mice, decreased constitutive Il-10 mRNA expression by 50% (Fig. 3.22D).
Figure 3.19 Effect of CDDP on body and liver weight in wild-type and Fgf21-null mice.

Adult male wild-type and age-matched male Fgf21-null mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment/genotype). Control received saline. Mouse body weights were monitored daily. After treatment, mouse livers were collected and weighted. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between control and CDDP treatment. Single daggers (†) represent a statistical difference (p < 0.05) between wild-type and Fgf21-null mice.
Figure 3.20 Effect of CDDP on liver injury in wild-type and Fgf21-null mice.

Adult male wild-type and age-matched male Fgf21-null mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment/genotype). Control received saline. Mouse liver were collected and processed for H&E staining. 40× magnification, (Scale bar: 200 μm). Details of liver with...
higher magnification shown below. Arrow, infiltrated immune cells; arrowhead, cytoplasmic vacuolation; circle, nuclear pyknosis and cellular degeneration.
Figure 3.21 Serum ALT and AST levels in wild-type and Fgf21-null mice following CDDP treatment.

Adult male wild-type and age-matched male Fgf21-null mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment/genotype). Control received saline. Mouse sera were collected and processed for ALT and AST measurement. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between control and CDDP treatment. Single daggers (†) represent a statistical difference (p < 0.05) between wild-type and Fgf21-null mice.
Figure 3.22 Effect of CDDP on Tnfa (A), Il-1β (B), Il-6 (C), and Il-10 (D) mRNA expression in wild-type and Fgf21-null mouse livers.

Adult male wild-type and age-matched male Fgf21-null mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment/genotype). Control received saline. Mouse livers were collected and
processed for mRNA analysis by RT-PCR. The data are presented as mean ± SEM.

Asterisks (*) represent a statistical difference \( (p < 0.05) \) between control and CDDP treatment. Single daggers (†) represent a statistical difference \( (p < 0.05) \) between wild-type and Fgf21-null mice.
3.3.2 Regulation of triglycerides (TG), total cholesterol, total bile-acid and glucose/glycogen levels in mouse sera and livers by CDDP in wild-type and Fgf21-null mice

FGF21 plays important roles in lipid and sugar metabolism (Ogawa et al., 2007; Chen, C et al., 2018). 3 and 16 mg/kg of CDDP decreased serum and liver TG levels in wild-type but not Fgf21-null mice (Table 3.1).

3 and 16 mg/kg of CDDP increased total cholesterol levels in wild-type mouse serum and livers (Table 3.1). Disruption of Fgf21 function, as observed in Fgf21-null mice, increased total cholesterol levels in mouse serum and livers (Table 3.1). However, CDDP did not alter total cholesterol levels in the serum and livers of Fgf21-null mice (Table 3.1).

Moreover, 3 and 16 mg/kg of CDDP increased total bile-acid levels in wild-type mouse serum and livers (Table 3.1). Disruption of Fgf21 function increased total bile acid levels in mouse serum and livers (Table 3.1). In Fgf21-null mice, 16 mg/kg of CDDP increased total bile-acid levels in serum but not in the livers (Table 3.1).

Furthermore, 16 mg/kg of CDDP increased serum glucose levels in both wild-type and Fgf21-null mice (Table 3.1). In addition, 16 mg/kg of CDDP decreased glycogen levels in wild-type but not Fgf21-null mouse livers (Table 3.1).
Table 3.1 Biochemical analysis of serum and livers of wild-type and Fgf21-null mice followed by CDDP treatment

<table>
<thead>
<tr>
<th></th>
<th>WT mice</th>
<th>Fgf21-null mice</th>
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<tr>
<td></td>
<td>CONT</td>
<td>CDDP (3 mg/kg)</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>36.79±1.01</td>
<td>16.71±2.22*</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>61.61±2.75</td>
<td>76.11±2.88*</td>
</tr>
<tr>
<td>Total BA (µM)</td>
<td>16.41±0.83</td>
<td>19.76±0.90</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>163.32±6.39</td>
<td>175.53±14.78</td>
</tr>
<tr>
<td>Triglyceride (mg/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>8.58±1.59</td>
<td>6.38±0.70</td>
</tr>
<tr>
<td>Total Cholesterol (mg/g)</td>
<td>3.67±0.07</td>
<td>4.05±0.16</td>
</tr>
<tr>
<td>Total BA (µmol/g)</td>
<td>0.35±0.02</td>
<td>0.45±0.02*</td>
</tr>
<tr>
<td>Glycogen (mg/g)</td>
<td>45.08±3.72</td>
<td>48.08±1.92</td>
</tr>
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</table>

Adult male wild-type and age-matched male Fgf21-null mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment/genotype). Control received saline. Mouse serum and livers were collected and processed for biochemical analysis. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between control and CDDP.
treatment. Single daggers (†) represent a statistical difference ($p < 0.05$) between wild-type and Fgf21-null mice.
3.3.3 DEX and β-naphthoflavone (BNF) attenuated progression of CDDP-induced hepatotoxicity via Fgf21 induction

We previously reported that DEX increased Fgf21 expression levels in mouse serum and liver (Vispute et al., 2017). We performed DEX-CDDP cotreatment to determine whether DEX can attenuate CDDP-induced liver injury via Fgf21 induction. Seven-day pre-treatment of DEX (2 mg/kg) increased Fgf21 mRNA expression by 5.1-fold in wild-type mouse livers (Fig. 3.23A). Four-day 16 mg/kg of CDDP treatment increased Fgf21 mRNA expression by 11-fold in wild-type mouse livers (Fig. 3.23A). Co-treatment of DEX and CDDP induced Fgf21 mRNA expression by 11.4-fold (Fig. 3.23A). Similarly, pre-treatment of 2 mg/kg DEX increased Fgf21 protein level by 1.5-fold in wild-type mouse livers (Fig. 3.23B). CDDP (16 mg/kg) increased Fgf21 protein level by 3-fold in wild-type mouse livers (Fig. 3.23B). Co-treatment of DEX and CDDP increased Fgf21 protein level by 2.8-fold (Fig. 3.23B). DEX increased serum Fgf21 protein levels (from 475 pg/ml to 660 pg/ml) (Fig. 3.23C). Compared to 2205 pg/ml of serum Fgf21 protein level after CDDP treatment (16 mg/kg), co-treatment of DEX with CDDP further increased serum Fgf21 protein level to 2303 pg/ml (Fig. 3.23C).

Morphologically, DEX did not apparently alter the body or liver weight, and pre-treatment of DEX recovered body and liver weight loss caused by CDDP (Fig. 3.24A and B). DEX did not cause apparent liver injury in wild-type mice, and evidenced by no alteration in liver histology (Fig. 3.24C and D), as well as no change of serum ALT or
AST levels (Fig. 3.25). DEX pre-treatment decreased liver injury and inflammation caused by CDDP in wild-type mouse livers (Fig. 3.24 and Fig. 3.25). As shown in Fig. 3.24E and F, hepatocytes were relieved from hypertrophy, dilatation and congestion in sinusoids; and the number of infiltrated immune cells (arrows) were decreased by DEX pre-treatment. In addition, DEX alleviated CDDP-increased serum ALT and AST levels (Fig. 3.25).

We further determined mRNA expression of several inflammation genes regulated by CDDP with or without DEX pre-treatment. DEX (2 mg/kg) did not alter any mRNA expression of any pro-inflammatory genes (Tnfα, Il-1β and Il-6), but decreased mRNA expression of Il-10), an anti-inflammatory gene (Fig. 3.26). CDDP (16 mg/kg) increased pro-inflammatory genes Il-1β and Il-6 by 3.3- and 3.7-fold, respectively in wild-type mouse livers (Fig 3.26B and C). CDDP (16 mg/kg) also increased anti-inflammation gene Il-10 mRNA expression by 3.4-fold in wild-type mouse livers (Fig. 3.26D). DEX pre-treatment alleviated CDDP-induced Il-1β and Il-10 mRNA expression (Fig. 3.26B and D).

β-naphthoflavone (BNF) is another FGF21 inducer (Cheng et al., 2014). To determine whether gain of Fgf/FGF21 function has protective effects in in vitro, DEX and BNF were applied to increase Fgf/FGF21 in cultured hepatoma cells. Pre-treatment of 1 µM DEX or 0.1 µM BNF improved cell viability following 1 µM of CDDP (Fig. 3.27).
A

Fgf21 mRNA expression (normalized to 18S)

CONT  DEX  CDDP  DEX+CDDP

B

Fgf21 (21 kDa)

β-actin (42 kDa)

Fgf21 protein (fold of β-actin)

CONT  DEX  CDDP  DEX+CDDP

C

Serum Fgf21 protein (pg/ml)

CONT  DEX  CDDP  DEX+CDDP
Figure 3.23 Co-treatment of DEX and CDDP on Fgf21 expression in mouse livers.

A. Regulation of Fgf21 mRNA expression; B. Representative immunoblotting images and quantitative presentation of Fgf21 protein level; C. Regulation of serum Fgf21 protein level following DEX and CDDP in mouse livers. Adult male C57BL/6 mice were pretreated with DEX (2 mg/kg) i.p. administration or control (corn oil) once daily for 7 days and then given CDDP at doses of (16 mg/kg) or control (saline) once daily for 4 days (n=6/treatment). Mouse livers were collected and processed for mRNA analysis by RT-PCR or membrane protein analysis by Western blots. 18S and β-actin were used as the loading control, respectively. Mouse sera were isolated for Fgf21 protein measurement by ELISA. The data are presented as means ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and treatment.
Figure 3.24 Effect of DEX pre-treatment on CDDP caused body and liver weight loss as well as CDDP-induced liver injury in mice.

A, Regulation of body weight; B, Regulation of the ratio of liver/body weight; C, H&E staining of mouse livers following DEX and CDDP treatment. 40× magnification, (Scale bar: 200 μm). Details of liver with higher magnification shown below. Arrow, infiltrated immune cells. Adult male C57BL/6 mice were pretreated with DEX (2 mg/kg) i.p. administration or control (corn oil) once daily for 7 days and then given CDDP at doses of (16 mg/kg) or control (saline) once daily for 4 days (n=6/treatment). Mice were weighted and mouse liver were collected, weighted and processed for histology analysis.
Figure 3.25 Alterations of serum ALT and AST levels following DEX and CDDP cotreatment in mice.

Adult male C57BL/6 mice were pretreated with DEX (2 mg/kg) i.p. administration or control (corn oil) once daily for 7 days and then given CDDP at doses of (16 mg/kg) or control (saline) once daily for 4 days (n=6/treatment). Mouse sera were collected and processed for measurement of ALT and AST levels. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and treatment. Single daggers (†) represent a statistical difference ($p < 0.05$) between combination (DEX+CDDP) and CDDP only groups.
Figure 3.26 Impact of co-treatment of DEX on Tnfα (A), Il-1β (B), Il-6 (C), and Il-10 (D) mRNA expression post-CDDP challenge in mouse livers.

Adult male C57BL/6 mice were pretreated with DEX (2 mg/kg) i.p. administration or control (corn oil) once daily for 7 days and then given CDDP at doses of (16 mg/kg) or control (saline) once daily for 4 days (n=6/treatment). Mouse livers were collected and
processed for mRNA analysis by RT-PCR. The data are presented as mean ± SEM.

Asterisks (*) represent a statistical difference ($p < 0.05$) between control and treatment.

Single daggers (†) represent a statistical difference ($p < 0.05$) between combination (DEX+CDDP) and CDDP only groups.
Figure 3.27 Cell viability analysis following co-treatment of DEX/BNF and CDDP in cultured mouse and human hepatoma cells.

Mouse Hepa1c1c7 (A) and human HepG2 (B) hepatoma cells were pre-treated with DEX (1 µM) or BNF (0.1 µM) for 24 hours. Then the cells were treated with 1 µM CDDP for another 48 hours. Control received 0.1% DMSO. Cells were processed for MTT assay. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and CDDP treatment. Single daggers (†) represent a statistical difference ($p < 0.05$) between cotreatment group (DEX+CDDP or BNF+CDDP) vs. CDDP-only group.
3.3.4 Gene regulation of lipid metabolism by CDDP in wild-type and Fgf21-null mouse livers

To explain serum and hepatic alterations of TG, total cholesterol and total bile acids by CDDP in wild-type and Fgf21-null mice, we further investigated the regulation of some lipid metabolism genes in mouse livers.

SREBP (sterol regulatory element binding protein) 1c and SREBP2 are important transcriptional factors regulating fatty acid and cholesterol biosynthesis, respectively (Guo et al., 2014). 16 mg/kg of CDDP decreased mRNA expression of SREBF (sterol regulatory element binding transcription factor) 1c (encoding SREBP1) by 85% and 90% in wild-type and Fgf21-null mouse livers, respectively (Fig. 3.28A). 16 mg/kg of CDDP decreased mRNA expression of SREBF2 (encoding SREBP2) by 84% in wild-type mouse livers (Fig. 3.28B). Disruption of Fgf21 function, as observed in Fgf21-null mice, decreased mRNA expression of SREBF2 by 90% (Fig. 3.28B). CDDP further decreased mRNA expression of SREBF2 in Fgf21-null mouse livers (Fig. 3.28B).

HMGCS (HMG-CoA synthase) and HMGCR (HMG-CoA reductase) are two important enzymes involved in cholesterol synthesis, in which HMGCR is the rate-limiting enzyme. CDDP decreased mRNA expression of HMGCS and HMGCR by 84% and 93% in wild-type mouse livers, respectively (Fig. 3.28C and D). Disruption of Fgf21 function decreased mRNA expression of HMGCS and HMGCR by 75% and 84%,
respectively (Fig. 3.28C and D). CDDP further decreased their mRNA expression in Fgf21-null mouse livers (Fig. 3.28C and D).

ABCG (ATP-binding cassette G sub-family member) 5/8 are sterol transporters that are responsible for biliary cholesterol excretion. 16 mg/kg of CDDP decreased mRNA expression of ABCG8 by 40% in wild-type mouse livers (Fig. 3.28F). Disruption of Fgf21 function decreased mRNA expression of ABCG5 and 8 by 89% and 78%, respectively (Fig. 3.28E and F). In Fgf21-null mice, CDDP further decreased mRNA expression of ABCG5 (Fig. 3.28E).

Cyp7a1 is the rate-limiting enzyme in the classic pathway of bile acid synthesis. 16 mg/kg of CDDP decreased mRNA expression of Cyp7a1 by 64% and 86% in wild-type and Fgf21-null mouse livers, respectively (Fig. 3.28G).

SHP (small heterodimer partner) plays important roles in the maintenance of cholesterol and bile acid homeostasis by inhibiting conversion of cholesterol into bile acids. 3 and 16 mg/kg of CDDP decreased mRNA expression of SHP by 51% and 90% in wild-type mouse livers, respectively (Fig. 3.28H). Disruption of Fgf21 function increased mRNA expression of SHP by 2.2-fold (Fig. 3.28H). However, CDDP further decreased mRNA expression of SHP in Fgf21-null mice (Fig. 3.28H).

NTCP (Na\(^+\)-taurocholate cotransporting polypeptide) and BSEP (bile salt export pump) are two transporters responsible for hepatic uptake of bile acids from sinusoidal blood and efflux of bile acids across the hepatocyte canalicular membrane into bile,
respectively. 16 mg/kg of CDDP decreased mRNA expression of NTCP and BSEP by 86% and 93% in both wild-type and Fgf21-null mouse livers (Fig. 3.18I and J).
<table>
<thead>
<tr>
<th>Gene</th>
<th>WT Mice</th>
<th>Fgf21-Null Mice</th>
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<tr>
<td><strong>Srebf1 mRNA expression (normalized to 18S)</strong></td>
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<td><a href="image">Graph</a></td>
</tr>
<tr>
<td><strong>Hmgcr mRNA expression (normalized to 18S)</strong></td>
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<tr>
<td><strong>Hmgcs mRNA expression (normalized to 18S)</strong></td>
<td><a href="image">Graph</a></td>
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<tr>
<td><strong>Abcg5 mRNA expression (normalized to 18S)</strong></td>
<td><a href="image">Graph</a></td>
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<tr>
<td><strong>Abcg8 mRNA expression (normalized to 18S)</strong></td>
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<tr>
<td><strong>Cyp7a1 mRNA expression (normalized to 18S)</strong></td>
<td><a href="image">Graph</a></td>
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<tr>
<td><strong>SHP mRNA expression (normalized to 18S)</strong></td>
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<tr>
<td><strong>Ntcp mRNA expression (normalized to 18S)</strong></td>
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<tr>
<td><strong>Bsep mRNA expression (normalized to 18S)</strong></td>
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Figure 3.28 Effect of CDDP on mRNA expression of lipid metabolism genes in wild-type and Fgf21-null mouse livers.

Adult male wild-type and age-matched male Fgf21-null mice were given i.p. administration of CDDP at doses of 3 and 16 mg/kg once daily for 4 days (n=6/treatment/genotype). Control received saline. Mouse livers were collected and processed for mRNA analysis by RT-PCR. The data are presented as mean ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between control and CDDP treatment. Single daggers (†) represent a statistical difference (p < 0.05) between wild-type and Fgf21-null mice.
CHAPTER 4 DISCUSSION

A recent study reported that FGF21 induction may protect against CDDP-induced acute kidney injury in mice (Li, F et al., 2018). In my dissertation, I further demonstrated that CDDP and TPA induced mouse and human hepatic Fgf/FGF21 expression through cJun-AP1 activation. In addition, Fgf/FGF21 induction can protect against the progression of CDDP-induced hepatotoxicity.

Specifically, I reported that CDDP dose-dependently induced mouse and human Fgf/FGF21 mRNA and protein expression (Fig. 3.1, 3.2, 3.3, 3.4, 3.5 and 3.6). In addition to mouse liver, CDDP also induced Fgf21 expression in mouse kidney and heart (Fig. 3.1, 3.2 and 3.4). Gain of Fgf21 function induced by DEX or BNF cotreatment attenuated, whereas loss of FGF21 function as depicted in Fgf21-null mice exaggerated CDDP-induced hepatotoxicity.

CDDP induced mouse and human Fgf/FGF21 mRNA expression in hepatoma cells only after 6 and 12 hours, but not 24 hours later (Fig. 3.1). Actually, AP-1 (c-Jun/c-Fos) is already known to be an immediate early gene, which is activated rapidly in response to cellular stress (Su et al., 2002). In addition, it is known that increased binding of AP-1 to its target gene promoters is an early response event after TPA and CDDP exposure (Li et al., 1998; Li et al., 1999). Therefore, CDDP may activate AP-1 and then rapidly induced FGF21 expression.

FGF21 actions require KLB and FGFRs (Ogawa et al., 2007). KLB is obligatory for
FGF21 to protect against high glucose induced oxidative stress and inflammation in primary mouse cardiomyocytes (Wu et al., 2017). Recent studies also showed that KLB deficiency can lead to lipotoxicity and inflammation in both mouse and human liver (Somm et al., 2018; Dongiovanni et al., 2020). Our results showed that CDDP induced Klb expression in a Fgf21-dependent manner in mouse liver (Fig. 3.7). In addition, only Fgfr1 but no other Fgfrs is induced by CDDP in mouse liver (Fig. 3.8). Several studies also suggested that FGF21 acts primarily through FGFR1 (Kharitonenkov et al., 2008; Kurosu et al., 2007). Therefore, in addition to FGF21, CDDP also induced the expression of KLB and FGFR1, two obligatory factors for FGF21 actions.

PGC1α is a common target gene of activation of PPARα and FGF21 signaling pathways (Domouzoglou et al., 2015; Liu et al., 2015). In our results, CDDP dose-dependently induced mRNA expression of Pgc1α in wild-type but not Fgf21-null mouse liver (Fig. 3.9A). CDDP did not activate PPARα signaling, evidenced by no induction of Cyp4a14, a characteristic PPARα target gene in mouse liver. Therefore, CDDP induced Pgc1α expression in a Fgf21-dependent manner.

We next investigated the mechanism responsible for CDDP-induced FGF21 expression. Previous studies reported that CDDP can produce endoplasmic reticulum (ER) stress and that ER stress can induce FGF21 expression via eIF2-ATF4 signaling (Wu et al., 2011; Kuo et al., 2016; Schaap et al., 2013). However, our studies showed that
CDDP did not apparently produce ER stress and did not activate ATF4 in mouse liver (Fig. 3.10A and B).

Other than ER stress, I showed that CDDP produced mitochondrial stress, evidenced by decreased mitochondrial respiration and ATP production in mouse and human hepatoma cells, as well as increased expression of GDF15, a biomarker of mitochondrial stress (Fig. 3.9B). Mitochondrial stress activates multiple signaling pathways, such as Nrf2 and JNK-cJun-AP1. CDDP did not apparently increase nuclear Nrf2 protein levels and did not apparently induce mRNA expression of Nqo1 and Ho-1, two biomarker genes of Nrf2 activation, in mouse liver (Fig. 3.10E, F, G and H). Therefore, in mouse liver, CDDP did not apparently activate Nrf2 signaling.

As we discussed above, CDDP activated AP-1. AP-1 is commonly activated following Mito stress-JNK-cJun-AP1 signaling (Rössler et al., 2017). My results showed that CDDP increased nuclear levels of phospho-JNK and phospho-c-Jun protein in mouse liver (Fig. 3.12). In addition, selective AP-1 inhibitor (SR11302) abolished CDDP-induced Fgf/FGF21 expression (Fig. 3.14), suggesting that AP-1 activation is required for Fgf/FGF21 induction by CDDP. In silico DNA sequence analysis showed that several putative AP1 response elements exist in the 2.5-kb promoter sequences of mouse and human Fgf/FGF21 genes. We then engineered mouse/human Fgf/FGF21 promoter sequences into pGL3 luciferase reporter vector and conducted Dual luciferase reporter assays (Promega; Fitchburg, WI). CDDP increased Fgf/FGF21 promoter activity in both
mouse Hepa1c1c7 and human HepG2 cells transiently transfected with 2.3-kb mouse Fgf21 or 2.0-kb human FGF21-pGL3 constructs. In contrast, CDDP-increased Fgf/FGF21 promoter activity was markedly attenuated after partial deletion of AP-1 binding sites (Fig. 3.15). Therefore, AP-1 response element located -1,347bp upstream of TSS in mouse Fgf21 gene promoter and AP-1 response elements located -1,140bp and -704bp upstream of TSS in human FGF21 gene promoter are essential for CDDP to induce Fgf/FGF21 expression (Fig. 3.15).

We next determined whether CDDP increased the binding of phospho-c-Jun protein to putative AP-1 binding sites in mouse and human Fgf/FGF21 gene promoter by performing ChIP-qPCR assays. CDDP increased the binding of phospho-c-Jun to DNA fragments located around -1,347bp and -499bp in mouse Fgf21 gene promoter, as well as DNA fragments located around -1,140bp and -704bp in human FGF21 gene promoter (Fig. 3.16).

Moreover, we confirmed that CDDP induces FGF21 expression via cJUN-AP1 activation in other human hepatoma cells. In the cells where CDDP induced FGF21 expression, c-JUN is also induced (Fig. 3.17). Whereas in the cells where CDDP did not apparently induce FGF21 expression, there is no c-JUN induction either (Fig. 3.17).

Furthermore, TPA, another known AP-1 activator, induced mRNA expression of Fgf/FGF21 and c-Jun/JUN in hepatoma cells. Such induction was attenuated by SR11302
co-treatment (Fig. 3.18). Taken together, we revealed that cJun-AP-1 activation is required for CDDP to induce Fgf/FGF21 expression.

Aminotransaminases, including ALT and AST, can release into the circulation after hepatocyte damage, which serve as the most sensitive indicators for liver injury (Işeri et al., 2007). A previous report showed that a single infusion of CDDP to a 47-year-old man with bladder carcinoma led to elevated AST level, hepatocellular ballooning necrosis, steatosis and mild cholestasis (Cavalli et al., 1978). But in general, CDDP clinically caused hepatotoxic only at relatively high doses. In addition, a high glucose level accompanied by low levels of insulin has been reported in a 43-year-old man with oropharyngeal squamous cell carcinoma after third cycle of CDDP treatment (once every four weeks), suggesting that CDDP therapy may worsen hyperglycemia (Goldstein et al., 1983; Komdeur et al., 2007). In mice, CDDP caused liver injury, evidenced by increased serum ALT and AST levels (Fig. 3.21), as well as histopathological changes of cellular degeneration, sinusoidal dilatation and inflammatory cells infiltration around portal area (Fig. 3.20). In addition, CDDP-induced liver injury is more severe in Fgf21-null than wild-type mice, indicating that FGF21 may protect against CDDP-induced liver injury.

CDDP increased total cholesterol, glucose and total bile acid levels in wild-type mouse serum (Table 3.1), which are consistent with previous reports in animals (Mousa-Al-Reza Hadjzadeh et al., 2017). In contrast, CDDP decreased serum levels of triglycerides (TG) in wild-type mice (Table 3.1). The discrepancy of TG levels between
our results and others may be due to the difference in mouse strains and dosing (Portilla et al., 2016), as well as a significant decreased in body mass and liver weight by CDDP in our current results (Fig. 3.19).

FGF21 is a metabolic regulator in lipid and sugar homeostasis (Ogawa et al., 2007; Chen, C et al., 2018). Disruption of Fgf21 function, as observed in Fgf21-null mice, increased total cholesterol and total bile-acid levels in mouse serum and livers (Table 3.1). SREBP2 is primarily responsible for activation of genes involved in cholesterol synthesis (Madison, 2016). Disruption of Fgf21 function, as observed in Fgf21-null mice, decreased mRNA expression of SREBP2, and other cholesterogenic genes, such as HMGCR and HMGCS (Fig. 3.28B, C and D). Meanwhile, CDDP decreased mRNA expression of ABCG5/8, preventing cholesterol excretion, as well as increased mRNA expression of SHP, inhibiting cholesterol conversion to bile acid, which may result in increased levels of total cholesterol (Fig. 3.28E, F, H and Table 3.1).

In Fgf21-null mice, 16 mg/kg of CDDP decreased serum TG levels (Table 3.1), which may be explained by down-regulation of SREBP1 by CDDP (Fig. 3.28A).

CDDP increased total cholesterol levels in serum and livers of wild-type mice (Table 3.1). In contrast, CDDP did not apparently alter total cholesterol levels in both serum and livers of Fgf21-null mice (Table 3.1). It may be explained by decreased mRNA expression of ABCG8 and Cyp7a1 following CDDP treatment, thus preventing
cholesterol transport and conversion into bile acids, and consequently increasing total cholesterol levels in wild-type mice (Fig. 3.28).

Furthermore, CDDP increased serum total bile acid levels in both wild-type and Fgf21-null mice (Table 3.1), which may be due to decreased expression of NTCP and BSEP by CDDP (Fig. 3.28I and J). CDDP decreased mRNA expression of BSEP in both wild-type and Fgf21-null mouse livers, which can lead to an increased level of total bile acids (Fig. 3.28J). However, CDDP only increased total bile acids in wild-type but not Fgf21-null mouse livers (Table 3.1). Therefore, many of other transporters or bile acid metabolic regulator still need to be further investigated.

In addition to organ toxicities, CDDP can cause nausea and vomiting in 90% of the patients (Locker et al., 2006). Glucocorticoids, such as dexamethasone (DEX) and methylprednisolone, are first-line antiemetic applied to improve the tolerance. The combination of DEX and CDDP has been established in clinical (Chu et al., 2014; Pufall, 2015). Our previous study revealed the protective role of DEX-induced FGF21 against APAP-induced liver injury (Vispute et al., 2017). In the present study, pre-treatment of DEX also attenuated CDDP-induced liver injury, evidenced by histopathological improvement, reduction of CDDP-increased serum ALT and AST levels, as well as attenuation of CDDP-induced inflammatory gene expressions in wild-type mice (Fig. 3.24 and Fig. 3.25). It should be noted that both DEX and FGF21 have anti-inflammatory effects, we cannot confirm whether inflammation is reduced by DEX or through FGF21
at this point. In addition, I also showed that gain of Fgf/FGF21 function following DEX/BNF co-treatment can relieve cytotoxicity of CDDP in cultured mouse and human hepatoma cells (Fig. 3.27).

Taken together, regulation of lipid and sugar homeostasis, as well as its anti-inflammation effects may contribute to FGF21’s cytoprotective roles against CDDP-induced hepatotoxicity.
CHAPTER 5 SUMMARY AND CONCLUSIONS

Over two decades, there are considerable achievements in the scientific understanding of the physiological, pharmacological and toxicological effects of FGF21 (Li et al., 2014; Lewis et al., 2019). Recently, a novel FGF21 analog, B1344, has been reported to improve fatty liver disease in nonhuman primates (Ye et al., 2013; Cui et al., 2020). Up to date, FGF21 has shown the benefits in metabolic regulation, anti-oxidation, anti-inflammation, treatment of metabolic diseases, and protection against drug/chemical-induced toxicities. A recent study reported that FGF21 may protect against CDDP-induced acute kidney injury (Li, F et al., 2018). However, the role of FGF21 in CDDP-induced liver injury and the underlying mechanism has not been studied. My dissertation work demonstrated that AP-1 activation can induce Fgf/FGF21 expression and that gain of Fgf/FGF21 function alleviates CDDP-induced liver injury.

In my dissertation, I firstly reported that CDDP increased Fgf/FGF21 expressions in both \textit{in vivo} mouse studies and \textit{in vitro} cell culture studies. In mouse livers, CDDP increased Klb and Fgfr1 expression in Fgf21-dependent manner, which are the co-receptor and the predominant receptor of Fgf21, respectively. In addition, CDDP induced the expression of Pgc1α in a Fgf21-dependent manner. It was recently reported that PGC1α can cooperate with Nrf2 to alleviate CDDP-induced oxidative stress in ovarian cancer cell line (Deng et al., 2020) and that Klotho proteins have potential metabolic, neuroprotective, cardioprotective, hepatoprotective and antitumor effects (Somm et al.,
2018; Dongiovanni et al., 2020; Baranowska et al., 2020). Therefore, the overall activation of FGF21/KLB/FGFR1-PGC1α signaling may have potential benefits against CDDP-induced hepat-, nephron-, or cardiotoxicity, which could be further investigated in the future research.

I also reported that CDDP induced Fgf/FGF21 expression via activation of mitostress-JNK-cJun-AP1 signaling pathway. However, it should be noted that a recent report showed that JNK signal pathway behaves as a double-edged sword in CDDP treatment, which is a pro-apoptosis factor but also can increase resistance to CDDP chemotherapy (Yan et al., 2016). Therefore, there should be a balance between tumor toxicity and hepatoprotection.

In addition, it should be interesting to future investigate the intricate molecular mechanisms of crosstalk between FGF21 and other signaling pathways to protect or mitigate drug-induced toxicities. For instance, a correlation between GDF15 and FGF21 has been reported in patients with mitochondrial diseases (Montero et al., 2016). Our results also showed CDDP induced GDF15 expression in FGF21-dependent manner. Another study showed that alcohol and carbon tetrachloride (CCl4) induced hepatic GDF15, which protected against alcohol and CCl4-induced liver damage in mice (Chung et al., 2017). Combined effect of FGF21 and GDF15 should be considered to investigate their potential synergistic therapeutic effects against chemical-induced hepatotoxicity.
We have previously reported that DEX (2 mg/kg) prevented APAP-induced hepatotoxicity, primarily via FGF21 induction (Vispute et al., 2017). My dissertation further showed cotreatment of DEX can also attenuate the progression of CDDP-induced hepatotoxicity.

In conclusion, my dissertation revealed that activation of cJun-AP1 signaling by CDDP and TPA, can induce mouse and human hepatic Fgf/FGF21 expression. In addition, gain of Fgf/FGF21 function can attenuate the progression of CDDP-induced hepatotoxicity.
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