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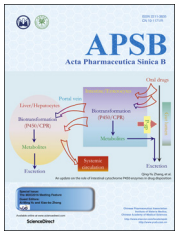

REVIEW

Crosstalk of HNF4α with extracellular and intracellular signaling pathways in the regulation of hepatic metabolism of drugs and lipids

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Abstract The liver is essential for survival due to its critical role in the regulation of metabolic homeostasis. Metabolism of xenobiotics, such as environmental chemicals and drugs by the liver protects us from toxic effects of these xenobiotics, whereas metabolism of cholesterol, bile acids (BAs), lipids, and glucose provide key building blocks and nutrients to promote the growth or maintain the survival of the organism. As a well-established master regulator of liver development and function, hepatocyte nuclear factor 4 alpha (HNF4α) plays a critical role in regulating a large number of key genes essential for the metabolism of xenobiotics, metabolic wastes, and nutrients. The expression and activity of HNF4α is regulated by diverse hormonal and signaling pathways such as growth hormone, glucocorticoids, thyroid hormone, insulin, transforming growth factor-β, estrogen, and cytokines. HNF4α appears to play a central role in orchestrating the transduction of extracellular hormonal signaling and intracellular stress/nutritional signaling onto transcriptional changes in the liver. There have been a few reviews on the regulation of drug metabolism, lipid metabolism, cell proliferation, and inflammation by HNF4α. However, the knowledge on how the expression and transcriptional activity of HNF4α is modulated remains scattered. Herein I provide comprehensive review on the regulation of expression and transcriptional activity of HNF4α, and how HNF4α crosstalks with diverse extracellular and intracellular signaling pathways to regulate genes essential in liver pathophysiology.

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1. Introduction

1.1. Overview of key biological functions of hepatocyte nuclear factor 4α (HNF4α)

HNF4α is a well-established master regulator of liver development and function. HNF4α is essential for hepatocyte differentiation and morphogenesis in fetal liver[1,2] and maintenance of liver function in adults[3–5]. Results from studies of adult mice with liver-specific knockout of *Hnf4α* demonstrate that HNF4α is essential in regulating hepatic expression of key genes in drug metabolism, bile acid synthesis and conjugation, lipid homeostasis, gluconeogenesis, ureagenesis, cell adhesion, as well as cell proliferation and apoptosis[3,6–11]. Hepatic expression and/or transcriptional activity of HNF4α is decreased markedly in non-alcoholic steatohepatitis, alcoholic liver disease, tumor necrosis factor-α (TNFα)-induced hepatotoxicity, severe cirrhotic livers, and hepatoma progression[12–16]. In contrast, ectopic expression of HNF4α in combination with the pioneering factor Foxa2 (HNF3β) in fibroblasts can induce the transdifferentiation of fibroblasts into hepatocyte-like cells[17]. Overexpression of HNF4α1 markedly inhibits liver carcinogenesis and liver fibrosis[18,19]. Mice implanted with human hepatoma cells that overexpress HNF4α1 have much longer survival, and intratumoral overexpression of HNF4α1 blocks tumor growth[20]. Thus, down-regulation of HNF4α is a major contributing factor to diverse liver diseases, such as steatohepatitis, liver fibrosis, and liver cancer, whereas restoration of HNF4α can inhibit liver cancer and improve liver function simultaneously. Currently, there is great interest in targeting HNF4α for stem-cell therapy and treatment of liver diseases such as liver cirrhosis and liver cancer. Nevertheless, HNF4α is an orphan nuclear receptor that lacks well-established activating ligands, although fatty acid thioesters have been reported as ligands of HNF4α[21]. Conversely, the expression and transcriptional activity of HNF4α is modulated by diverse extra- and intracellular signaling pathways, and various transcriptional factors can physically interact with HNF4α to regulate hepatic gene expression. There have been a few reviews on the role of HNF4α in regulation of drug metabolism, lipid metabolism, cell proliferation, and inflammation[5,22–24]. However, the knowledge on how the expression and transcriptional activity of HNF4α is modulated remains scattered. Herein I summarize the modulation of hepatic expression and transcriptional activity of HNF4α by diverse extra- and intracellular signaling pathways, as well as how HNF4α crosstalks with various transcriptional factors to dictate hepatic expression of genes important in drug metabolism, lipid homeostasis, and cell proliferation.

1.2. HNF4α isoforms

There are two types, 9 isoforms of HNF4α transcripts resulting from alternative splicing and/or usage of 2 promoters, with 6 "adult" isoforms (4α1–α6) from the P1 promoter, but 3 "fetal" isoforms (4α7–α9) from the P2 promoter. P2 promoter–driven fetal HNF4α isoforms are expressed throughout liver development, but disappear after birth, whereas P1 promoter–driven adult HNF4α isoforms are abundant postnatally. Deregulation of HNF4α is a marker of epithelial tumor progression[25]. There is a remarkable switch in mRNA and protein expression from P1 to P2 promoter–driven HNF4α in transgenic livers and hepatocellular carcinoma (HCC) of EGF-overexpressing transgenic mice and human HCC[26]. Interestingly, HNF4α inhibits the P2 promoter activated by HNF6 and HNF1α[27]; thus, dynamic changes in HNF4α isoform expression may be self-regulated by HNF4α1. Importantly, the "adult" HNF4α1 and "fetal" HNF4α7 have different transactivation properties, namely, HNF4α7 more efficiently activates promoters of early hepatocyte genes (such as α-fetoprotein), whereas HNF4α1 has a more significant impact on genes of main hepatic differentiation markers[28]. Targeted deletion of the *Hnf4α1* isoform in mouse liver results in liver steatosis and marked down-regulation of constitutive androstane receptor (Car), a key xenobiotic receptor[29]. Overexpression of HNF4α2 decreases, whereas overexpression of HNF4α8 increases the invasiveness of colon cancer cells[17]. Currently, the mechanism of dynamic switch of HNF4α1/4α2 and HNF4α7/4α8 expression during liver development and carcinogenesis remains unknown.

1.3. Regulation of gene expression by HNF4α

The P1 HNF4α proteins, such as HNF4α1 and HNF4α2, have two activation domains, namely activation function-1 (AF-1) and AF-2 which synergize for full HNF4α transactivation activity. The N-terminal AF-1 (A/B) domain and C-terminal AF-2 domain convey the transactivation activity of HNF4α, whereas the C-terminal F-domain of HNF4α exhibits repressor activity (Fig. 1A)[30]. The P2 HNF4α isoforms, such as HNF4α8 lack the N-terminal AF-1 domain and thus generally have much weaker transactivation activity for HNF4α-target genes. Different from other nuclear receptors, HNF4α binds to DNA as a homodimer, and the interaction between its ligand binding domain (LBD) and DNA-binding domain (DBD) (Fig. 1A) is essential for the high DNA-binding affinity of the homodimer[31]. In a study in human colon cancer cells, HNF4α2 was found to have many more DNA-binding sites than HNF4α8, although they have the identical DBD with a conserved double zinc finger motif[32]. HNF4α generally binds to direct repeat 1 (DR1) or DR2 site in the promoter and recruits co-activators to transactivate its target genes[33,34]. In addition to direct regulation of mRNA gene expression, HNF4α can transactivate microRNA-29; *Hnf4α*-deficiency in mouse liver down-regulates miR-29, resulting in induction of miR-29–target gene DNA methyltransferase 3 and epigenetic reprogramming[35]. HNF4α can repress gene expression *via* recruiting the co-repressor silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and histone deacetylase to the promoter, leading to epigenetic silencing of target genes[28]. Loss of HNF4α in young-adult mouse liver markedly altered epigenome, manifested by global increases in key histone modifications such as histone H3 lysine-4 trimethylation (H3K4me3), H3K27me3, and H3K9me2, which is associated with induction of the corresponding epigenetic enzymes in *Hnf4α*-deficient liver[36]. Thus, regulation of epigenome appears to be a key mechanism of regulation of the transcriptome and liver development by HNF4α.

2. Factors modulating HNF4α activity

2.1. Modulation of the transcriptional activity of HNF4α by fatty acids

Different from many other nuclear receptors that require ligands and retinoid X receptor α (RXRα) as an obligatory heterodimerization partner for transactivation, HNF4α is constitutively active and bind to DNA as a homodimer[37]. The LBD of HNF4α is responsible for the selectivity of binding partner (homodimer

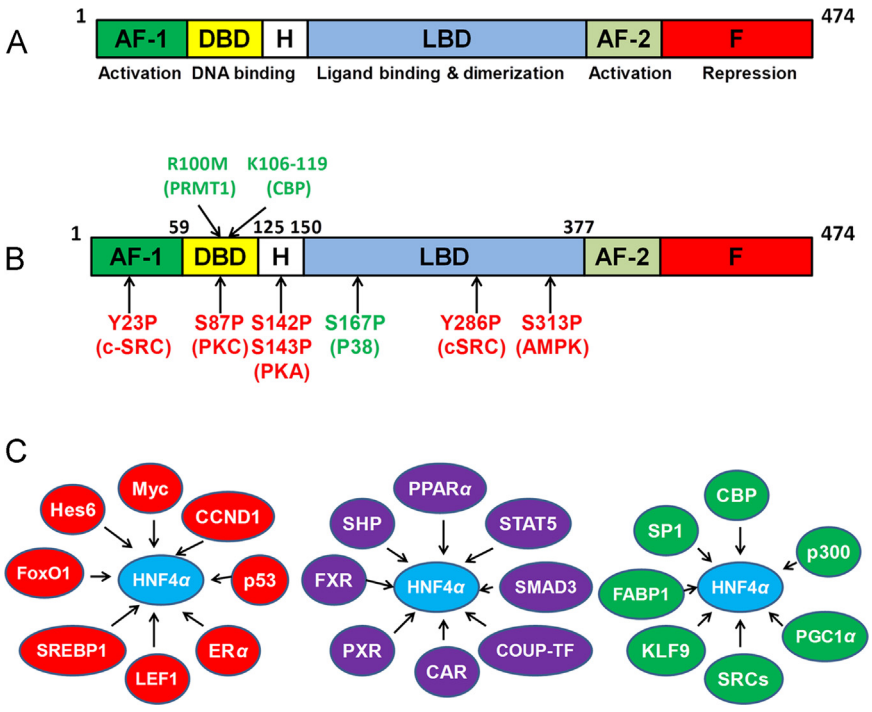

Figure 1 Diagrams that illustrate the protein domain structure (A), posttranslational modifications (B), and interactions (C) of HNF4α with other signaling pathways. (A) Domain structure of HNF4α protein, with the 474-amino-acid-long human HNF4α2 shown as the canonical HNF4α isoform. (B) Posttranslational modifications of HNF4α. HNF4α is methylated at arginine 100 (R100M) by PRMT1, and acetylated at lysines 106, 108, 118, or 119 by CBP. HNF4α is phosphorylated at lysine-23 (Y23P) and Y286 (Y286P) by c-SRC, serine 87 (S87P) by PKC, serine 142 and 143 (S142P and S143P) by PKA, serine 167 (S167P) by P38, and serine 313 (S313P) by AMPK. The positions of post-translational modifications of HNF4α have been renumbered in the text and Fig. 1 based on the updated NCBI protein database for HNF4α2 (NP_000448.3), which is also used as the canonical protein isoform for human HNF4α in PhosphoSitePlus®, a public database for posttranslational modifications of proteins[43]. (C) Transcriptional factors that modulate the transcriptional activity of HNF4α through physical interactions. Red shape: negative interaction; purple shape: both negative and positive interactions; green shape: positive interaction.

versus heterodimer)[37]. The X-ray crystal structure of an HNF4α protein fragment that contains the HNF4α LBD but lacks the transactivation F-domain shows that the ligand binding pockets of both the closed and open forms contain fatty acids[38]. However, occupancy of the ligand linoleic acid does not appear to significantly affect the HNF4α transcriptional activity[39]. The conversion of fatty acids to fatty acyl-CoAs by fatty acyl-CoA synthetases is required for the modulation of HNF4α transcriptional activity by fatty acids[21]. Using His-tagged full-length HNF4α protein, GST-tagged LBD of HNF4α, and radio-labeled fatty acyl-CoAs, fatty acyl-CoA thioesters were found to bind to the LBD of HNF4α with high affinity and selectivity over peroxisomal proliferator-activated receptor α (PPARα) and RXRα[21]. The effects of saturated fatty acids on HNF4α are dependent on chain length: (C16:0) acid activates whereas (C18:0) acid suppresses HNF4α transcriptional activity, whereas saturated fatty acids shorter than C16 are inactive. In contrast, unsaturated long-chain fatty acids dose-dependently suppress HNF4α transcriptional activity[21]. Interestingly, shorter chain (C14:0 and C16:0) fatty acyl-CoA markedly enhances, whereas long-chain (C18:0 or C18:3, ω-3) fatty acyl-CoA markedly decreases the binding of HNF4α protein to its cognate enhancer DNA[21]. Conversely, HNF4α has thioesterase activity[40], which might be a mechanism of feedback regulation. Acyl-CoA-binding protein and liver type fatty acid binding protein (L-FABP) physically interact with HNF4α, slow the degradation of fatty acyl-CoA, and potentiate the transactivation of target genes by HNF4α[41]. Dietary supplementation of medium-chain triglycerides preserved HNF4α expression and improved alcohol-induced hepatic lipid dyshomeostasis in rats[12]. Thus, further understanding the mechanism of regulation of HNF4α transactivation activity by various fatty acids and their acyl-CoA thioesters may help develop novel approaches to activate HNF4α to treat liver and metabolic diseases.

2.2. *Post-translational modifications of HNF4α*

2.2.1. *Methylation*

The arginine methyltransferase PRMT1 methylates arginine-100 (R100) in the DBD of HNF4α to enhance the affinity of HNF4α for its binding site (Fig. 1B)[42,43]. Moreover, facilitated by the p160 family of coactivators, PRMT1 is recruited to the HNF4α LBD and methylates histone H4 at arginine 3, leading to nucleosomal alterations and subsequent RNA polymerase II preinitiation complex formation[42]. Analysis of the crystal structure of the HNF4α homodimer/DNA complex showed that the methylation of arginine-100 would more firmly "glue" the DBD junctional interface with both LBDs[31].

2.2.2. *Acetylation*

Acetylation of HNF4α at lysine residues within the nuclear localization sequence of DBD by CREB-binding protein (CBP)

is essential for nuclear localization of HNF4α and transactivation of HNF4α-target genes (Fig. 1B)[43,44].

2.2.3. Phosphorylation

During inflammation-redox stress induced by combined treatment of hepatocytes with interleukin 1β (IL-1β) and H_2O_2, phosphorylation of HNF4α at serine-167 (S167) in LBD by p38 mitogen-activated protein kinase (MAPK) is essential for the interaction of HNF4α with the co-activator PC4 to induce the expression of inducible nitric-oxide synthase[45]. Phosphorylation of HNF4α by the p38 is important for the protein stability and nuclear levels of HNF4α in hepatocytes[46]; however, phosphorylation at S167 by p38 is not required for the induction of cytochrome P450 7A1 (CYP7A1) by HNF4α in hepatocytes, suggesting that p38 might phosphorylate HNF4α at more than one site[46].

The c-SRC tyrosine kinase markedly inhibits the activity of P1, but not P2 products of HNF4α *via* selective phosphorylation of P1 HNF4α proteins at tyrosine 23 (Y23) and 286 (Y286), which correlates with isoform-specific loss of HNF4α in human colon cancer[47]. Phosphorylation of HNF4α at S87 within DBD by protein kinase C (PKC) decreased the DNA binding, transactivation ability, and protein stability of HNF4α[48]. In contrast, starvation decreased DNA-binding of HNF4α to the promoter of L-type pyruvate kinase, a glycolytic enzyme in rat liver *via* cAMP-PKA-phosphorylation of HNF4α at S142 and S143[49]. Interestingly, the cAMP-mediated regulation of HNF4α depends on the level of the co-activator PPARγ coactivator 1α (PGC1α)[50]: cAMP/PKA inhibited the transcriptional activity of HNF4α in COS-1 cells, whereas a stimulatory effect in HepG2 cells is dependent on the induction of PGC1α by cAMP in HepG2 cells[50]. HNF4α and PGC1α are induced in mouse liver during fasting[51]. Thus, the effects of HNF4α phosphorylation by PKA may be gene- and cell-context dependent. The AMP-activated protein kinase (AMPK) is the central component of a cellular signaling system. AMPK inhibits the transcriptional activity of HNF4α *via* direct phosphorylation of HNF4α at S313 in the LBD, leading to decreased formation of homodimer and accelerated degradation of HNF4α protein[52].

2.3. Interaction with co-activators

PGC1α (Fig. 1C), which also has acetyltransferase activity, is a key co-activator of HNF4α for the transactivation of certain gluconeogenic genes such as phoenolpyruvate carboxykinase (*PEPCK*) and glucose-6-phosphatase (G-6-Pase)[51]. However, many other HNF4α-target genes are not co-activated by PGC1α[4]. Co-activators steroid receptor coactivator-1 (SRC-1), SRC-2, and SRC-3 also enhance the transcriptional activity of HNF4α[53,54].

2.4. Physical interaction with co-repressors

2.4.1. Interaction with Hes family bHLH transcription factor 6 (Hes6)

Hes6 is a direct transcriptional target of HNF4α; hepatic *Hes6* mRNA expression is largely down-regulated in *Hnf4α*-null mice[55]. Conversely, Hes6 inhibits the transactivation of *Hnf4α* promoter by PPARα, and Hes6 forms a complex with HNF4α during the fed state to inhibit the expression of certain HNF4α-target genes[55]. Hes6 alone cannot directly bind to DNA. Hes6 physically interacts with HNF4α protein and displace coactivators PGC1α and CBP from HNF4α[55]. During fasting, hepatic Hes6 expression is markedly down-regulated, and the HNF4α–Hes6 complex in the promoters of fatty acid metabolism–associated genes is replaced by the activated PPARα, resulting in gene induction[54]. Thus, Hes6 negatively regulates the HNF4α and PPARα signaling. In mouse liver, Hes6 can be induced by retinoic acid receptor in response to its natural agonist ligand all-trans retinoic acid[56]. It remains unknown whether Hes6 acts as a general or gene-specific co-repressor of HNF4α.

2.4.2. Interaction with small heterodimer partner (SHP)

Without a DBD, the orphan nuclear receptor SHP mainly functions as a co-repressor by interacting with a large number of transcription factors[57]. Overexpression of SHP causes fatty liver, whereas SHP is a tumor suppressor in liver[57]. Interestingly, HNF4α appears to play a key role in mediating the nuclear translocation of SHP: in 293T cells, the exogenously expressed SHP protein is localized in both the cytosol and nucleus; ectopic expression of HNF4α results in exclusive nuclear translocation of the SHP protein[58]. Interestingly, the interaction of HNF4α with SHP markedly increases nuclear and total cellular levels of both HNF4α and SHP proteins, likely due to increased protein stability[59]. SHP can interact with the AF-2 domain of HNF4α to prevent the recruitment of co-activator SRC-3 to inhibit the transactivation of certain HNF4α-target genes[54]. Co-activators SRC-1 and SRC-2 also interact with the AF-2 domain of HNF4α[53], and the coactivator CBP interacts with both the AF-1 and AF-2 domains of HNF4α[44], whereas the coactivator PC4 and members of the basal transcriptional machinery interact with the AF-1 domain of HNF4α[45]. Thus, the effect of SHP on the transactivation activity of HNF4α is most likely gene- and co-activator–specific. miR-34a is markedly induced in livers from mice deficient in farnesoid X receptor (FXR), and the FXR activator GW4064 down-regulates miR-34a in obese mouse liver *via* inducing SHP to inhibit p53 occupancy at the miR-34a promoter[60]. It appears that SHP might play an important role in regulating cellular protein levels of HNF4α by increasing HNF4α protein expression (*via* down-regulating miR-34a) and stabilizing HNF4α protein (*via* physical interaction). It remains unknown whether deficiency of *HNF4α* and *SHP in vivo* alters endogenous protein levels and cellular localization of each other. In this regard, *Hnf4α* deficiency causes marked induction of a large number of genes in mouse liver[61]; however, the underlying mechanism remains poorly understood. Future studies on the effects of *HNF4α* and *SHP* deficiency on the cellular levels and localization of each other will provide mechanistic insights on the role of SHP in modulating the gene regulation and anticancer action by HNF4α in the liver.

2.5. Crosstalk with nuclear receptors

2.5.1. Crosstalk with FXR

The role of BAs as hormones has been recognized since the discovery of BAs as ligands for the nuclear receptor FXR[62]. The pregnane X receptor (PXR), a key xenobiotic receptor, is also activated by BAs[63]. HNF4α is required for FXR expression in the fetal liver but not in the adult liver[2]. HNF4α can activate the human PXR promoter by binding to the DR1 motif in hepatoma cells[64]. In mice, HNF4α is essential for the expression of PXR in fetal liver by binding to the *PXR* promoter[65]. However, *Hnf4α* deficiency does not affect hepatic expression of PXR in adult mouse liver, and DNA-binding activity of PXR is enhanced in

Hnf4α-deficient young-adult mouse liver[10]. Interestingly, *PXR* is a FXR target gene in mouse liver[66]. FXR is likely activated in the adult *Hnf4α*-null liver by the accumulation of BAs during cholestasis. Thus, it is proposed that activation of FXR might prevent the down-regulation of PXR in adult *Hnf4α*-null livers[10]. Interestingly, FXR plays a key role in mediating the marked alterations of drug-processing genes (DPGs) in the long-lived growth hormone–deficient *lit/lit* mice; loss of FXR largely attenuated hepatic induction of *Cyp2b9*, *Cyp2b10*, *Cyp4a10*, *Cyp4a14*, flavin containing monooxygenase 3,3′-phosphoadenosine 5′-phosphosulfate synthase 2, cytochrome P450 oxidoreductase, sulfotransferase 1d1 (*Sult1d1*), UDP glucuronosyltransferase 1a1 (*Ugt1a1*), and ATP-binding cassette, sub-family B, member 1a (*Mdr1a*) in *lit/lit* mice[67]. Of these FXR-regulated genes, *Cyp2b9*, *Cyp4a14*, *Ugt1a1*, and *Mdr1a* are induced in male *Hnf4α*-null livers[10,68].

FXR co-immunoprecipitates with HNF4α in mouse liver[33], and FXR physically interacts with HNF4α in *in vitro* GST pulldown experiments[69]. ChIP-seq analysis showed that nearly 50% binding sites of FXR and HNF4α in mouse liver overlap[33]. Moreover, genes co-bound by FXR and HNF4α are enriched in drug metabolism and PPARα signaling pathway[33]. The DNA-binding of FXR to certain target genes, such as SHP and fibroblast growth factor 15, is enhanced in the *Hnf4α*-null mouse livers, and HNF4α antagonizes the transactivation of mouse *SHP* promoter by FXR[33]. Conversely, bile acids activate the FXR/RXR heterodimer to displace HNF4α from the promoter to down-regulate APOC3 in mouse liver and human hepatocytes[70]. In contrast, HNF4α potentiates the activation of the intron 1 of scavenger receptor class B type 1 by FXR[33]. Thus, FXR and HNF4α can cooperate or antagonize the activity of each other, suggesting that both factors can compensate for each other's deficiency at certain sites, and such compensation might be an important mechanism to maintain cellular integrity and homeostasis in liver diseases. Understanding how FXR signaling is altered in *Hnf4α*-deficient liver will provide new insights on the regulation of drug metabolism and homeostasis of bile acids and lipids during *Hnf4α* deficiency.

2.5.2. *Crosstalk with PPARα*

In addition to HNF4α, PPARα is another key regulator of hepatic metabolism of drugs and lipids. The promoter of *PPARα* can be transactivated by HNF4α and PPARα itself[71], and *Hnf4α* deficiency down-regulated PPARα in adult mouse liver[3]. HNF4α and PPARα share DR1 as the common consensus DNA-binding site[72]. Interestingly, both antagonism and cooperation between HNF4α and PPARα have been reported. Both HNF4α and PPARα can bind to the DR1 sites in the promoters of acyl-CoA oxidase and acyl-CoA thioesterase I, and PPARα is a much stronger transactivator than HNF4α; consequently, HNF4α suppressed the gene-activating function of PPARα on these two genes due to competition for a common binding site[73,74]. In contrast, HNF4α and PPARα cooperate to induce multifunctional protein 1, one of the most abundant proteins in murine peroxisome[75]. Activation of PPARα is required for the marked down-regulation of Na^+-taurocholate cotransporting polypeptide (NTCP), an HNF4α-dependent key BA uptake transporter, by perfluorodecanoic acid in mouse liver[76]. Interestingly, certain PPARα target genes, such as genes of L-FABP and microsomal triglyceride transfer protein (MTTP)[77] were markedly down-regulated, whereas three PPARα target genes, such as genes of carnitoyl-palmitoyl transferase-II, MCAD, and 3-hydroxy-3-methylglutaryl CoA synthase were markedly induced in *Hnf4α*-null mouse livers[3]. Such differential changes in PPARα target genes in *Hnf4α*-null livers may be due to the down-regulation of PPARα as a strong transactivator, the loss of HNF4α as a competitor for PPARα, or the induction of co-activator of PPARα, such as PPAR-binding protein in *Hnf4α*-null livers[10].

2.5.3. *Crosstalk with PXR and CAR*

PXR and CAR are key xenobiotic receptors that regulate hepatic expression of a large number of DPGs[78]. HNF4α transactivates hepatic expression of PXR and CAR[65,79], and HNF4α synergizes with PXR and CAR to induce PXR- and CAR-target DPGs[80,81]. Interestingly, there is a functional inhibitory cross-talk between CAR and HNF4α in hepatic lipid/glucose metabolism. CAR down-regulates HNF4α-target genes through competing for common coactivators and/or competing with HNF4α for binding to DR1 motif in the promoter of *Cyp7a1*, the rate-limiting enzyme in bile-acid biosynthesis[82]. Accordingly, the CAR activator TCPOBOP decreased hepatic expression of *Cyp7a1* and *Cyp8b1* in mice. Additionally, PXR also inhibits the expression of *CYP7A1*, likely due to the competition of PXR with HNF4α for the common coactivator PGC1α[83]. Interestingly, activation of PXR promotes drug metabolism but causes hepatosteatosis, whereas activation of CAR increases drug metabolism and attenuates steatosis[84]; differential effects of PXR and CAR activation on hepatic expression of lipogenic genes may be the underlying mechanism[85].

2.5.4. *Crosstalk with estrogen receptor α (ERα)*

ERα suppresses the HNF4α-transactivation of HBV enhancer I *via* ERα–HNF4α physical interaction which is independent of DNA-binding by ERα[86]. Hepatic ERα expression is stimulated by elevated blood levels of estrogen[86]. Results from studies of *Erα*-null mice demonstrate that ERα is essential in mediating estrogen-induced cholestasis by down-regulating certain genes essential in the transport and synthesis of bile acids[87], some of which, namely *Ntcp*, *Oatp1a1*, *Cyp7a1*, and *Cyp8b1* are HNF4α-target genes[9,10]. The potential role of ERα–HNF4α interaction in the down-regulation of DPGs by estrogen during cholestasis warrants investigation.

2.6. *Physical interaction with other transcription factors*

2.6.1. *Interactions with chicken ovalbumin upstream promoter transcription factors (COUP-TFs)*

The effects of orphan nuclear receptors COUP-TFI and COUP-TFII on the transcriptional activity of HNF4α are promoter dependent. COUP-TFs negatively affect gene transcription by competing with HNF4α in binding to the common binding site (*e.g.*, DR1 site) within gene promoters of aldehyde dehydrogenase 2, hepatic lipase, apoA-I, apoA-II, apo-B, and apoC-III[88–90]. In contrast, HNF4α and COUP-TFII synergistically induce *CYP7A1* by binding to the adjacent different sites within the promoter of *CYP7A1*[91]. Conversely, COUP-TFs do not directly bind to the *HNF1α* promoter; instead, COUP-TFs physically interact with the LBD of HNF4α to markedly enhance the transactivation of *HNF1α* promoter by HNF4α[92].

2.6.2. *Interactions with specificity protein 1 (SP1), c-Myc, and cyclin D1 (CCND1)*

In addition to activation of target genes through binding to DR1/DR2 sites, HNF4α can interact with the general transcription factor

SP1 to induce p21, which is independent of DNA-binding of HNF4α[93]. The protooncogene c-Myc can compete with HNF4α in interacting with the promoter-bound SP1 to block the induction of p21 in hepatoma cells[93]. Moreover, c-Myc competes with HNF4α for the control of apolipoprotein C3 (APOC3)[93]. Additionally, CCND1 inhibits hepatic lipogenesis *via* inhibiting the activity of the carbohydrate response element-binding protein (ChREBP), and CCND1 binds to HNF4α protein to inhibit the recruitment of HNF4α to the promoter of lipogenic genes in hepatocytes[94]. CCND1 also inhibits the transcriptional activity of PPARγ, a key lipogenic nuclear receptor, and *Ccnd1*-null mice have fatty liver[95]. Knockout of *Hnf4α* in livers of adult chow-fed mice markedly induced c-Myc, CCND1, and hepatocyte proliferation which was associated with fatty liver but decreased blood levels of triglycerides and cholesterol[6]. Interestingly, adenoviral overexpression of CCND1 in rat liver induced robust cell proliferation and marked alterations in hepatic mRNA expression of a large number of DPGs and lipogenic genes[96]. Thus, interaction of CCND1 with HNF4α may play important roles in the regulation of the metabolism of drugs and lipids during liver development and liver injury repair.

2.6.3. *Interaction with p53*

The p53 protein is a well-established tumor suppressor. Hepatic mRNA expression of p53 is much higher in perinatal liver than adult liver in mice, and p53 ranks as a top upstream regulator of target genes during liver development[97]. p53 is activated in steatotic livers in patients, and inhibition of p53 by pifithrin-α *p*-nitro attenuates steatosis and liver injury in a mouse model of non-alcoholic fatty liver disease[98]. Moreover, inhibition of p53 protects liver tissue against endotoxin-induced apoptotic and necrotic cell death in rats[99]. The p53 protein inhibits *HNF4α* transcriptional activity *via* interacting with its LBD and recruiting histone deacetylase[100]. Moreover, p53 down-regulates *HNF4α* mRNA expression by binding to the P1 promoter of *HNF4α*[101]. Furthermore, the p53-target miR-34a is a potent inhibitor of the protein expression of HNF4α[102]. Thus, p53 appears to be a powerful multifaceted inhibitor of HNF4α. A fine-tuned balance between p53 and HNF4α may be important in maintaining the homeostasis of the liver during liver development and injury repair.

2.6.4. *Interaction with β-catenin in "metabolic zonation"*

Metabolic zonation, manifested by differential expression of metabolic genes in the periportal (PP) and perivenular (PV) hepatocytes, is a key feature of differentiated mature liver. The basal and xenobiotic-induced expression of the main phase I and phase II drug-metabolizing enzymes is confined to the PV hepatocytes[103]. The Wnt/β-catenin pathway is essential for both the proliferation and differentiation of hepatocytes during liver development. Spontaneous differentiation of liver stem cells gives rise to PP hepatocytes that, after Wnt pathway activation, switches into PV hepatocytes[104]. Hepatocyte-specific deletion of β-catenin causes the loss of "metabolic zonation", manifested by the dramatic down-regulation of certain DPGs such as *Cyp1a2*, *Cyp2c*, and *Cyp2e1*[105]. HNF4α plays a dual role in regulating metabolic zonation by activating PP genes but suppressing PV genes in PP hepatocytes[106]. The Wnt downstream player LEF1 interacts with HNF4α to displace HNF4α from its own consensus site to suppress the expression of PP genes in the liver[104]. In *Hnf4α*-deficient adult mouse liver, the Wnt/β-catenin pathway is strongly activated[107]. Importantly, β-catenin interacts with different co-factors to exert different biological activities. The CDP/β-catenin-mediated transcription is critical for proliferation, whereas the p300/β-catenin-mediated transcription initiates differentiation[108,109]. Interestingly, CDP is a key co-activator of HNF4α, whereas the interaction of p300 with HNF4α is essential to potentiate the transactivation of HNF1-target genes[110]. Thus, *Hnf4α*-deficiency may alter the interaction of co-activators CDP and p300 with β-catenin, resulting in marked deregulation of β-catenin signaling and accelerated cell proliferation and dedifferentiation of hepatocytes.

2.6.5. *Interaction with thyroid hormone-responsive Kruppel-like factor 9 (KLF9)*

The prohormone T4 can be catalyzed by type 1 iodothyronine deiodinase (Dio1) to form the active T3, whereas T3 can be inactivated by Dio3. The high ratio of Dio3 to Dio1 in fetal liver keeps T3 at low levels in the fetal circulation[111]. Upon birth, there is a surge in blood levels of T4 and T3 which stimulate gluconeogenesis in the liver[111]. Thyroid dysfunction profoundly alters the expression of many key drug metabolizing enzymes and transporters in liver, kidney, and intestine[112–115]. Thyroid hormone (TH) is also important in regulation of hepatic lipid metabolism[116]. TH potently induces P450 oxidoreductase (POR) in HepG2 cells and rat liver[117,118]. TH increases HNF4α mRNA and protein levels in HepG2 cells[119]. KLF9, a GC box-binding protein of SP1 family transcription factors, regulates certain cytochrome P450 genes, such as *CYP1A1*, *CYP2D6*, and *CYP7A1*, by binding to the CACCC core sequence in the promoter[120–122]. KLF9 is induced by TH receptor in mouse and human hepatocytes; the induction of KLF9 by T3 in neonatal human hepatocytes is much stronger than in adult hepatocytes[123]. KLF9 plays a key role in modulating the response of HepG2 cells to T3[123]. Interestingly, KLF9 synergizes with HNF4α to induce human *CYP2D6*[122] and mouse *Dio1*[124]. However, no direct physical interaction of KLF9 and HNF4α can be detected[122], whereas the physical interaction of GATA4 with both HNF4α and KLF9 appears to be essential for synergistic activation of *Dio1* gene by HNF4α and KLF9[124]. Hepatic mRNA expression of KLF9 increases during postnatal development, whereas KLF9 is down-regulated in liver cancer, and overexpression of KLF9 inhibits the proliferation of liver cancer cells[125]. Thus, KLF9 appears to play an important role in liver differentiation and maturation promoted by TH and HNF4α. In contrast, KLF9 promotes lipogenesis in adipocytes and hepatocytes[126,127], and KLF9 mediates acetaldehyde-induced c-Jun N-terminal kinase (JNK)-dependent alphaI(I) collagen gene expression in hepatic stellate cells (HSCs)[128]. *Hnf4α* deficiency in liver markedly elevates blood levels of T4[124] and rapidly causes fatty liver and liver fibrosis. Thus, it will be interesting to determine how *Hnf4α* deficiency may alter the expression and biological activities of KLF9 in hepatocytes and HSCs and its contribution to fatty liver and liver fibrosis.

2.6.6. *Interaction with transforming growth factor-beta (TGF-β) and SMAD*

The TGF-β signaling pathway is essential in the regulation of different cellular processes, including proliferation, differentiation, migration or cell death, which is essential for tissue homeostasis. TGF-β signaling participates in all stages of liver disease progression, from initial liver injury through inflammation and fibrosis, to cirrhosis and cancer[129]. TGF-β promotes liver differentiation during embryogenesis and physiological liver regeneration by

exerting cytostatic and apoptotic effects on hepatocytes[130]. Interestingly, TGF-β and HNF4α rank among the top 3 upstream regulators of gene expression in postnatal liver development in mice[97]. TGF-β plays a dominant role in suppressing the function of HNF4α by transcriptional inhibition and posttranslational modification of HNF4α[131,132]. TGF-β activates its membrane receptor, which leads to phosphorylation and activation of Smad2 and Smad3 that can partner with the common mediator Smad4, and these heteromeric complexes can translocate to the nucleus to regulate specific gene expression[129]. In human hepatocytes, the TGF-β–activated Smad3/Smad4, but not Smad2, physically interact with HNF4α to synergistically induce apolipoprotein C3[133,134]. In contrast, interaction of the TGF-β–activated Smad3 with HNF4α inhibits the binding of HNF4α to the *CYP7A1* promoter and the activation of *CYP7A1* in human hepatoma and primary hepatocytes[135]. The DNA-binding of Smad2/Smad3 is highly cell-type-specific. In a genome-wide study, HNF4α-binding motif is identified as an enriched motif in the HepG2-specific Smad2/3 binding regions, and 32.5% of the Smad2/3 binding regions overlap HNF4α bindings[136], which illustrates an extensive crosstalk of Smads with HNF4α and an important role of HNF4α in dictating the cell-specific role of TGF-β. In hepatocytes, TGF-β signaling *via* Smad2 (which does not directly interact with HNF4α) promotes steatohepatitis through inducing cell death and lipogenesis in mice[137]. In contrast, results from 3D co-culture of hepatocytes and NIH3T3 cells demonstrate that TGF-β is required for the enhanced hepatocyte function of drug metabolism[138]. Taken together, there is extensive interaction of HNF4α with TGF-β during liver development and disease progression.

2.6.7. *Interaction with growth hormone (GH) and the JAK2/STAT5 pathway*

The essential role of GH in regulating body growth and maturation of the liver is well demonstrated by studies of *GH* transgenic mice[139] and the *Gh*-deficient *lit/lit* mice which have a spontaneous mutation in the growth-hormone releasing-hormone receptor[140–142]. Pituitary GH secretion pattern in humans and other species is highly pulsatile. In rodents, this pattern is sexually dimorphic; males have regular high-amplitude pulses and relatively low interpulse GH levels, and females have lower amplitude pulses and higher interpulse levels. In rodents, pulsatile or continuous GH increases or decreases STAT5b activation, respectively. The intermittent pulses of liver STAT5 activity are first observed at puberty (5 weeks of age in rats), when plasma GH pulsation first begins and expression of male-specific, GH pulse-activated liver genes first occurs[143]. Hepatic expression of drug metabolizing enzymes and transporters are profoundly altered in lit/lit mice[142,144–146], demonstrating a critical role of GH in regulating hepatic expression of DPGs. Disruption of GH-JAK2-STAT5 signaling is associated with liver diseases, including fatty liver, fibrosis, and liver cancer[147]. In contrast, activation of the JAK2-STAT5 pathway is a key driving force in the pathogenesis of myeloproliferative neoplasms and inflammation[148]. Thus, the biological role of STAT5 is highly cell-context-dependent. The interaction of HNF4α with GH-Jak2-Stat5 pathway plays a key role in coordinating gender-specific expression of DPGs in mice[11,149,150]. Interestingly, STAT5b and HNF4α exhibit bi-directional crosstalk which may enhance HNF4α-dependent gene transcription but inhibit STAT5b transcriptional activity *via* the inhibitory effects of HNF4α on JAK2 phosphorylation, leading to inhibition of STAT5b signaling initiated by the GH receptor at the cell surface[149]. Thus, HNF4α might play an important role in dictating the differential biological effects of STAT5 activation (anticarcinogenic in hepatocytes but procarcinogenic in hematopoietic cells). Currently, there is no report on how *HNF4α* deficiency alters STAT5 signaling in the liver.

GH secretion patterns are frequently disturbed during chronic diseases. Chronic kidney disease (CKD) is associated with resistance to the growth-promoting and anabolic actions of GH, leading to retardation of body growth in children and contributing to muscle wasting in adults[151]. In CKD rats, GH-induced tyrosine phosphorylation and nuclear translocation of STAT5 is markedly impaired[152]. Interestingly, the DNA-binding of HNF4α is markedly decreased in livers of CKD rats, which is associated with striking hepatic down-regulation of male-predominant CYP2C11 and CYP3A2[153]. Hyperlipidemia and decreased drug metabolism/disposition are characteristics of CKD patients[154,155], in which the disruption in the interaction between HNF4α and GH-JAK2/STAT5 pathway may have a key pathogenic role.

2.6.8. *Interaction with glucocorticoids and glucocorticoid receptor (GR)*

Glucocorticoids are essential in postnatal liver development. Results from studies of mice with liver-specific knockout of *Gr* demonstrate that GR function in hepatocytes is essential to promote postnatal body growth[156,157]. Additionally, direct GR–STAT5 interaction in hepatocytes is essential in the control of postnatal body growth and liver maturation[156]. Combined deficiency in hepatic STAT5 and GR signaling in *Stat5-Gr*-double-knockout mice increases hepatic lipid load and HCC formation[158]. Mice lacking GR in hepatocytes are indistinguishable from their littermates until 3–4 weeks of age, when the GH-dependent body growth becomes essential[157]. Interestingly, GR physically interact with STAT5 to function as an essential co-activator of STAT5 in mediating hepatic GH signaling to activate the transcription of genes essential for postnatal body growth[156]. Although both *Stat5* knockout and *Stat5/Gr* double knockout mice develop hepatosteatosis, only the *Stat5/Gr* double knockout mice develop inflammation and spontaneous liver tumors[158]. It is shown that GR binding to the enhancer of *Hnf4α* may induce hepatic expression of *Hnf4α* around birth in mice[159]. HNF4α and GR cooperate to induce hepatic expression of phosphoenolpyruvate carboxykinase[160], a key gene in gluconeogenesis, and they synergistically transactivate *CYP2A6*[161]. Additionally, induction of HNF4α by GR in human hepatocytes leads to induction of organic cation transporter 1 (*OCT1*)[162]. There is no report on whether HNF4α and GR can physically interact to co-regulate gene expression.

2.6.9. *Interaction with insulin-responsive transcription factors*

The transcription factors sterol regulatory element-binding proteins (SREBPs) are activated by insulin to promote lipogenesis and inhibit gluconeogenesis. Insulin resistance is often associated with hyperinsulinemia. During hyperinsulinemia, HNF4α is down-regulated by SREBP2 in mouse liver and human hepatocytes[163]. Through interaction of the transactivation domain of SREBP1 with the ligand binding/AF2 domains of HNF4α, SREBP1 competitively inhibits PGC1α recruitment by HNF4α, resulting in hepatic down-regulation of gluconeogenic genes *PEPCK* and *G6PC*[164]. The transcription factor FoxO1 interacts with the DBD of HNF4α to inhibit the binding of HNF4α to the cognate DNA; phosphorylation of FoxO1 by the insulin-PI3K pathway reverses the repression of HNF4α transcriptional activity by FoxO1[165]. Thus,

insulin appears to have a dual role in regulating the transcriptional activity of HNF4α *via* SREBPs and FoxO1.

3. Factors modulating HNF4α expression

3.1. Down-regulation by inflammation/infection and metabolic stresses

During inflammation, interleukin-1β (IL-1β) down-regulates HNF4α *via* the MAPK kinase (MEK)-1/2 and JNK MAPK signaling pathways in HepG2 cells and mouse liver[166]. A time-course study showed that *HNF4α* mRNA expression was decreased more than 80% 2–4 h after IL-1β treatment, but returned to control levels 12 h after IL-1β treatment in HepG2 cells[166]. Tumor necrosis factor α (TNFα) also down-regulates HNF4α expression *via* the JNK pathway in HepG2 cells[167]. Additionally, TNFα activates the NF-κB pathway to suppress the transcriptional activity, but not the expression, of HNF4α in hepatocytes[168]. Interestingly, HNF4α was shown to exert anti-inflammatory effects in human hepatocytes *via* the miR-124-IL6R-STAT3 pathway; knockdown of *HNF4α* in human hepatocytes leads to down-regulation of miR-124, induction of *IL6R* and *IL6*, and activation of STAT3[169]. However, there is no induction of IL-6 or activation of STAT3 in adult mice with acute loss of HNF4α[6]. Thus, there may be species difference between humans and mice regarding the interaction of HNF4α with inflammation in the liver. Additionally, HNF4α is down-regulated by hepatitis B virus (HBV) X protein by unknown mechanism[170,171]. The effects of HCV infection on HNF4α are less clear. *HNF4α* has been shown to be induced by the HCV infection and the HCV non-structural protein NS5[172,173], whereas HNF4α protein was reported to be reduced in HCV-infected hepatocytes and hepatoma cells due to the targeting of the 3′ UTR of *HNF4α* mRNA by the HCV-derived small non-coding RNA vmr11[174].

3.2. Inhibition of protein expression of HNF4α by microRNAs

The protein expression of HNF4α in hepatocytes is markedly inhibited by miR-21, miR-24, and miR-34a[16,102,175]. miR-34a is highly induced in patients with non-alcoholic steatohepatitis, diabetic mice, and mice fed a high-fat diet[16]. Overexpression of miR-34a reduces HNF4α expression and promotes liver steatosis and hypolipidemia[16]. The expression of miR-24 and miR-34a is markedly induced by the PKC/MAPK and reactive oxygen species pathways[102], whereas the oncomiR miR-21 is overexpressed in alcoholic liver injury, liver fibrosis, and liver cancer[175-177]. Thus, induction of miR-21, miR-24, and miR-34a during inflammation and metabolic/oxidative stresses may contribute to the posttranscriptional down-regulation of HNF4α in the liver.

3.3. Post-translational regulation by G protein Gα12

The G protein Gα12 is overexpressed in many types of cancers including liver cancer[178]. Transient expression of Gα12 only decreased HNF4α protein levels, whereas stable expression of Gα12 substantially decreased both the mRNA and protein levels of HNF4α in the human hepatoma Huh7 cells[179]. In Huh7 cells, activated Gα12 increased the ubiquitination and degradation of HNF4α protein[179]; however, the underlying mechanism remains unknown.

4. Knowledge gaps in understanding the role of HNF4α in regulation of hepatic gene expression and pathophysiology

Although much have been known about HNF4α, there are still some important knowledge gaps regarding the role of HNF4α in regulating hepatic metabolism of drugs and lipids.

4.1. Differential role of HNF4α in regulation of DPGs in mice and humans

In human livers, the mRNA expression of HNF4α correlates with a large number of DPGs[180]. The causative role of HNF4α in regulating DPGs in human liver has been studied by knocking down *HNF4α* in human hepatocytes with siRNA or viral vectors. Knockdown of *HNF4α* in human hepatocytes causes global down-regulation of DPGs and certain xenobiotic receptors, including CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, UGT1A1, UGT1A9, SULT2A1, ABCB1, ABCB11, ABCC2, OATP1B1 and OCT1, as well as those of PXR and CAR[181]. CYP2E1 plays important roles in the metabolism of environmental chemicals, ethanol, and therapeutic drugs such as acetaminophen[182]. Knockdown of *HNF4α* did not affect *CYP2E1* mRNA expression in primary human hepatocytes[181]. In contrast, knockdown of *HNF4α* markedly decreases CYP2E1 expression in HepG2 cells[170]. It is noteworthy that *HNF4α* mutation causes diabetes which can induce CYP2E1[182], and *Hnf4α*-deficiency in adult mouse liver activates the Wnt/β-catenin pathway, a key transactivator of *Cyp2e1*[105]. Thus, the role of HNF4α in the regulation of CYP2E1, a highly inducible enzyme, is not conclusive and may be cell context-dependent.

Sulfation is essential for the metabolism of hormones and detoxification of bile acids[183]. SULT2A1 is important in the sulfation of androgen precursor hormone dehydroepiandrosterone, bile acids, and hydroxymethyl polycyclic aromatic hydrocarbon procarcinogens[184]. HNF4α plays a central role in the control of SULT2A1 transcription by directly binding to the promoter of *SULT2A1* in human hepatocytes[185]. In humans, the expression of SULT2A1 is very low in fetal liver, but rapidly increases to high levels at 1 year after birth and remains little changed afterwards; there is no gender difference in hepatic expression of SULT2A1 in humans[186]. In contrast, in mice, hepatic mRNA expression of *Sult2as* peaks around weaning in both genders, after which hepatic expression of *Sult2as* sharply decreases to undetectable levels in male mice, but decreases to moderate levels in female mice, due to suppressive effects of androgens and male-pattern GH secretion, as well as stimulatory effects by estrogens and female-pattern GH secretion[187]. In parallel, serum and urinary levels of BA sulfates are high in humans but very low in adult male mice[188,189]. In mice, *Hnf4α* deficiency increased hepatic expression of *Sult2a2* in male mice[10], but moderately decreased *Sult2a* in female mice[11]. The effect of HNF4α on the promoter activities of mouse *Sult2as* has not been reported. There appears to be significant species difference between humans and adult mice regarding the role of HNF4α in regulating hepatic *Sult2a* expression as well as sulfation of hormones and BAs, which may have significant implications in the extrapolation of data from mice to humans. It remains to be determined whether the neonatal and adolescent male mice, which have similarly high hepatic expression of *Sult2as*, may be closer to humans regarding the role of HNF4α in regulating *Sult2as* and sulfation of hormones and BAs.

4.2. Knowledge gap regarding the role of HNF4α in regulation of lipid metabolism, inflammation, and cell death

The conversion of cholesterol to bile acids for biliary excretion is a major pathway for the excretion of excess cholesterol. A common feature in patients with chronic cholestatic liver disease is hyperlipidemia manifested by a marked increase of low-density lipoprotein (LDL) and variable high-density lipoprotein (HDL) cholesterol levels[190]. In contrast, in adult mice with liver-specific knockout of *Hnf4α*, the severe cholestasis is associated with markedly lower blood levels of triglycerides and more than 50% lower blood cholesterol than the wild-type mice[3]. Likewise, knockdown of *Hnf4α* in adult mouse liver also result in markedly decreased blood levels of triglycerides and cholesterol[191]. There are no signs of apoptosis or inflammation in *Hnf4α*-deficient livers of adult mice[6]. The marked deceases in blood levels of triglycerides and cholesterol and lack of increases in apoptosis and inflammation in *Hnf4α*-deficient livers of adult mice is in a sharp contrast to the hyperlipidemia and increases in apoptosis and inflammation in most chronic liver diseases such as alcoholic and non-alcoholic steatohepatitis, cholestatic liver injury, viral hepatitis, and liver cirrhosis, diseases in which HNF4α is often markedly down-regulated in both patients and animal models[12–15]. Thus, there is apparently a key knowledge gap regarding the role of HNF4α in the regulation of lipid metabolism, cell death, and inflammatory responses during chronic liver diseases. Is such discrepancy due to a partial loss of HNF4α in humans and mice with liver diseases versus a nearly complete loss of HNF4α in *Hnf4α*-null mouse livers? Alternatively, is such discrepancy due to the differences in the causes of *Hnf4α* deficiency, namely environmental insults (by inflammatory cytokines, viral proteins, ethanol metabolites, etc.) *versus* genetic deletion? It is noteworthy that various hormones and cytokines are dysregulated in chronic liver diseases. As aforementioned, these hormones and cytokines crosstalk extensively with HNF4α in the liver. Hepatocytes and hepatoma cells are resistant to LPS-induced cell death. Knockdown of *HNF4α* in immortalized human hepatocytes decreases apoptosis[169]. Interestingly, the dedifferentiated hepatoma cells that have been selected for the loss of the liver-enriched HNF4α/HNF1α are very sensitive to LPS-induced apoptosis[192]. In this regard, targeted deletion of *Hnf4α* in mouse intestines, where the epithelial cells are exposed to LPS released from the gut bacteria, increases both cell proliferation and apoptosis[193]. Moreover, when challenged with dextran sulfate sodium, mice with intestine-specific knockout of *Hnf4α* have markedly more severe colitis, manifested by the absence of epithelium and intensive submucosal infiltration of inflammatory cells[194]. Thus, loss of HNF4α might provide cells the proliferative and survival advantage under particular conditions, but might make cells more susceptible to cell death induced by inflammation and/or metabolic stresses.

Alcohol consumption and fat ingestion are closely associated and stimulated by each other[195]. Ethanol and fat consumption act synergistically to increase blood triglycerides levels[195]. Alcohol-induced hypertriglyceridemia is due to increased fat intake and VLDL secretion, impaired lipolysis, and increased free fatty acid fluxes from adipose tissue to the liver[195,196]. In contrast, the model of *ad libitum* feeding with the Lieber–DeCarli diet that contains ethanol, equal amount of fat (~30%–35% kcal fat) but much less carbohydrate for 4 weeks has been widely used in animal models of alcoholism; this model only induces mild steatosis and slight elevation of serum ALT, with little or no liver inflammation[197]. In comparison, feeding mice high-fat diet causes hepatic inflammation without hepatosteatosis[198], whereas mice fed a high-fat ethanol-containing diet followed by single dose of LPS injection develop severe steatohepatitis manifested by marked elevation of blood ALT, hepatic necrosis, accumulation of lipids, induction of inflammatory cytokines such as TNFα and IL-1β, characteristics that mimic human alcoholic steatohepatitis[199].

Hepatic HNF4α activity is decreased in mice by chronic ethanol consumption, partly due to the depletion of zinc as a key cofactor for HNF4α[15]. In view of the inhibitory effects of ethanol, high-fat, and inflammatory cytokines on the expression and/or activity of HNF4α, it is conceivable that HNF4α is markedly down-regulated in human alcoholic and non-alcoholic steatohepatitis[16]. It is likely that the marked hypolipidemia and lack of apoptosis or inflammation in the adult regular-chow-fed mice with liver-specific knockout of *Hnf4α* might not reflect the role of HNF4α in the regulation of lipid metabolism, apoptosis, and inflammation in patients and animal models of alcoholic or non-alcoholic steatohepatitis, when profound interactions among high-fat intake, LPS exposure, and/or ethanol are factored in. Interestingly, after challenged with the hepatic carcinogen diethylnitrosamine (DEN), adult *Hnf4α*-deficient mice have more liver tumors, which is associated with increases in inflammatory foci[61]. Future studies of mice with liver-specific knockout of *Hnf4α* under these stress conditions (*e.g.*, high-fat diet, ethanol consumption, and endotoxin challenge) may unveil surprising novel roles of HNF4α in the regulation of lipid metabolism, inflammation, and cell death in alcoholic and non-alcoholic steatohepatitis.

4.3. Knowledge gap in developmental-stage-specific effects of HNF4α deficiency on liver transcriptome and pathophysiology

Targeted deletion of *Hnf4α* in fetal mouse livers results in dramatic down-regulations of a large number of liver-enriched transcription factors, such as HNF1α, HNF1β, HNF3β, HNF6, liver receptor homolog-1 (LRH-1), FXR, PXR and CAR[2]. In contrast, targeted deletion of *HNF4α* in young-adult mouse livers results in only moderate down-regulations of HNF1α and HNF3β, no changes in FXR and PXR, but induction of HNF1β and LRH-1[2]. Loss of *Hnf4α* in fetal liver blocks the induction of proteins required for cell junction assembly and adhesion[200], resulting in the failure of morphological and functional differentiation of hepatocytes[201]. *Hnf4α*-deficient fetal liver has dramatic down-regulation of glycogen synthase GYS2 and key gluconeogenic enzymes glucose-6-phosphatase, catalytic subunit (G6PC) and phosphoenolpyruvate carboxykinase (PCK1), and much lower glycogen[201]. However, there are no changes in apoptotic cell death or cell proliferation between *Hnf4α*-null and control fetal livers[201]. In contrast, loss of HNF4α in adult mouse liver causes rapid cell proliferation, which is associated with activation of β-catenin and induction of c-Myc and cyclin D1, key factors in cell proliferation[6,107]. However, there is no increase of apoptosis, resulting in marked hepatomegaly in *Hnf4α*-null mice[6]. Thus, loss of HNF4α in fetal and adult liver causes distinct changes in hepatic transcriptome and cell proliferation; the underlying mechanism remains poorly understood.

Little is known about the role of HNF4α in postnatal liver development and maturation. There is a remarkable metabolic switch during postnatal liver development. The expression of most DPGs are very low in fetal liver[202]. Upon birth, there is an

immediate need for the clearance of metabolic waste and xenobiotics. Consequently, there is a postnatal surge in hepatic expression of DPGs right after birth[97,202,203]. In humans, hepatic expression of most DPGs reach near-adult levels by 1 year of age[202], whereas hepatic DPG expression in mice approaches adult levels shortly after weaning[97]. The remarkable postnatal changes in DPG expression is associated with marked alterations in energy and lipid metabolism. In utero, the main energy substrate transferred across the placenta is glucose[204]. However, after birth there is a sudden change of energy substrate to fatty acids due to the consumption of high-fat, low-carbohydrate milk, and this is associated with marked hepatic induction of the fatty-acid receptor PPARα and its target genes, such as *Cyp4a14/10*, acyl-CoA thioesterases, and *Cpt1a* during suckling[205]. Interestingly, a recent genome-wide analysis of inducible transcriptome by *PPARα* in human hepatocytes demonstrates a novel role of PPARα in inducing key DPGs such as *CYP3A4* and *CYP2C8* in humans[85]. The suckling-weaning transition is also accompanied by a change of the major energy source back to carbohydrates due to the intake of higher-carbohydrate and lower-fat solid foods. Thus, the postnatal peri-weaning period represents a key unique developmental stage for hepatic expression of genes essential in drug and lipid metabolism, in which the activation of PPARα and its extensive crosstalk with HNF4α[72] may play a major role. Currently, little is known about the role of HNF4α in postnatal liver development. In neonatal (1–2 weeks of age) double transgenic mice with *HBV* transgene, *Hnf4α* floxed at exons 4 and 5, and the Alb-cre, *Hnf4α* deficiency is associated with 50% lower body weight, hypoglycemia, elevated serum bile acids, and markedly decreased viral replication and viral RNA load[206]. Thus, HNF4α is essential for postnatal liver development and HBV replication; however, the role of HNF4α in regulating hepatic expression of DPGs and lipid metabolism during postnatal liver development remains largely unknown. We found that targeted deletion of *Hnf4α* in the neonatal peri-weaning mice markedly altered hepatic transcriptome and lipid metabolism, with some key changes highly distinct from those in mice with either fetal-liver- or adult-liver-specific knockout of *Hnf4α* (unpublished results). Understanding the mechanism of the neonatal/peri-weaning-specific role of HNF4α in regulating DPGs and lipid metabolism is important in developmental pharmacology.

4.4. Knowledge gap in differential effects of HNF4α mutations on plasma lipid profiles in humans

There are conflicting reports regarding the association of *HNF4α* mutations with blood lipid profiles in diabetic patients who carry various *HNF4α* mutations. In a maturity-onset diabetes of the young (MODY1) family with a nonsense mutation (R154X) of *HNF4α*, there is a paradoxical 3.3-fold increase in serum levels of lipoprotein(A)[207], which consists of an LDL-like particle and the specific apolipoprotein(A) (ApoA). Lipoprotein(A) [Lp(A)] levels are also elevated in three Japanese patients with MODY1 *HNF4α* mutations[208]. Type 2 diabetic patients with a loss-of-function T130I *HNF4α* mutation have lower blood levels of HDL cholesterol[209]. In contrast, 24 members of the HNF4α/MODY1 pedigree (Q268X mutation) have decreased blood levels of Lipoprotein(A) and triglycerides[210]. Moreover, 6 young MODY1 patients in Sweden have decreased blood levels of VLDL and LDL but slightly elevated HDL[211]. Currently, the mechanism of the differential effects of different *HNF4α* mutations on blood lipid profiles remains unknown. Although some confounding factors, such as individual variations, dietary factors, or drug treatments, may contribute to such discrepancy, mutations of *HNF4α* at different sites have been shown to exert differential effects on their transactivation activity, cellular localization, and the interaction with wild-type (WT) HNF4α and the co-repressor SHP. The R154X mutant lacks the E domain but retains DNA binding activity *in vitro;* R154X mutant has markedly decreased transactivation activity and exerts dominant-negative effects on WT HNF4α in β-cells[212]. In contrast, the Q268X mutant contains an intact DBD but a truncated dimerization domain and LBD; the Q268X mutant does not bind to DNA or form dimer, and it does not exert dominant-negative effect on WT HNF4α in HepG2 cells[213]. Interestingly, the R154X mutant lacks the binding ability to WT HNF4α or SHP, whereas the Q268X mutant can interact with and alter the cellular distribution of WT HNF4α and SHP [214]. It remains to be determined whether the putative differential effects of Q268X and R154X mutations on the mutant and WT HNF4α as well as SHP may cause divergent changes in lipid metabolism in MODY1 patients.

5. Conclusions and future perspectives

After intensive studies of HNF4α in the past two decades, much have been known about the importance of HNF4α in liver pathophysiology. HNF4α is critical in regulating all key aspects of liver development and function, with particular importance in regulating hepatic expression of DPGs and genes essential for the metabolism of cholesterol, bile acids, and lipids. The expression and activity of HNF4α are regulated by diverse extracellular and intracellular signaling pathways, and HNF4α crosstalks extensively with other transcription factors to dictate hepatic gene expression. Thus, HNF4α sits in the center of the hierarchy of liver transcriptional network to coordinate various extra- and intra-cellular signaling to fine-tune the liver transcriptome during hepatocyte proliferation, differentiation, and maturation. *HNF4α* mutations cause MODY1 in humans, whereas reduced expression and/or activity of HNF4α is associated with all major liver diseases, such as alcoholic and non-alcoholic steatohepatitis, viral hepatitis, liver cirrhosis, and liver cancer. Although the activation ligands for HNF4α have not been definitively identified, it is very encouraging that overexpression of *HNF4α* mRNA in hepatoma and/or the cirrhotic liver can not only inhibit liver cancer but also improve liver function and ameliorate liver cirrhosis. This means that approaches that boost the mRNA or protein expression of HNF4α can not only inhibit liver cancer but also improve liver function, which is highly desirable for the treatment of liver cancer, a deadly disease that lacks effective pharmacological treatment.

Despite decades of intensive research, there are still some important knowledge gaps regarding how the expression and activity of HNF4α is regulated, and how the deficiency of HNF4α may differentially affect gene expression and pathophysiology under various infectious/inflammatory and metabolic stresses. It remains to be determined whether the various *HNF4α* mutations and posttranslational modifications (*e.g.*, methylation, acetylating, and phosphorylation) of HNF4α and co-activators/co-repressors cause uniform or gene-specific changes in the expression of HNF4α-target genes. *HNF4α* deficiency results in hepatic induction of a large number of genes; however, the mechanism of suppression of gene

expression by HNF4α remains poorly understood. In view of the extensive crosstalk of HNF4α with other signaling pathways, many of which have a dual role in modulating liver pathophysiology, deficiency of *HNF4α* will likely shift the balance of these signaling pathways toward detrimental outcomes. For example, the JAK/STAT5 pathway can promote inflammation[147], and the TGF-β/Smad2, but not the TGF-β/Smad3 pathway, promotes steatohepatitis in hepatocytes[136]. Thus, it is important to understand how *HNF4α* deficiency alters these signaling pathways so that we can better understand the pathogenesis induced by *HNF4α* deficiency. Some MODY1 patients develop young-onset diabetes before puberty, and HNF4α is likely down-regulated in various inflammatory and viral liver diseases in children. How *HNF4α* deficiency in neonates and adolescence affects liver pathophysiology, particularly the metabolism of drugs and lipids, is an important knowledge gap to be bridged in pediatric pharmacology. To fully understand the impact of *HNF4α* deficiency on the liver and the whole body, *Hnf4α*-null mice need to be challenged with various stresses (*e.g.* viral infection, inflammation, high-fat diet, and xenobiotic treatment). Lastly, the development of novel approaches that effectively enhance the expression and/or activity of HNF4α may provide very promising novel therapy for liver diseases, particularly liver cancer.

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