Nuclear receptors pregnane X receptor (PXR) and constitutive active/androstane receptor (CAR) are xenobiotic-responsive transcriptional factors that belong to the same subfamily and are expressed abundantly in the liver. They play crucial roles in various liver functions including xenobiotic disposition and energy metabolism. CAR is also involved in xenobiotic-induced hepatocyte proliferation and hepatocarcinogenesis in rodents. However, there are some open questions on the association between chemical carcinogenesis and these nuclear receptors. These include the molecular mechanism for CAR-mediated hepatocyte proliferation and hepatocarcinogenesis. Another important question is whether PXR is associated with hepatocyte proliferation. We have recently reported a novel and unique function of PXR associated with murine hepatocyte proliferation: PXR activation alone does not induce hepatocyte proliferation but accelerates hepatocyte proliferation induced by various types of stimuli including CAR- or peroxisome proliferator-activated receptor alpha activating compounds, liver injury, and growth factors. We have also reported a role of yes-associated protein (YAP), a transcriptional cofactor controlling organ size and cell growth under the Hippo pathway, in CAR-mediated hepatocyte proliferation in mice. In this review, I will introduce our recent results as well as related studies on the roles of PXR and CAR in xenobiotic-induced hepatocyte proliferation and their molecular mechanisms.

Key words  nuclear receptor; hepatocyte proliferation; chemical carcinogenesis; gene regulation; protein–protein interaction

1. INTRODUCTION

Pregnane X receptor or PXR (also known as NR1I2), and constitutive active/androstane receptor or CAR (also known as NR1I3), are members of the nuclear receptor gene superfamily and xenobiotic-responsive transcription factors. Both receptors are highly expressed in the liver and intestines and play pivotal roles in protection of the body from harmful xenobiots through upregulating genes encoding drug metabolizing enzymes and transporters such as CYP3A and CYP2B subfamily members of CYPs, uridine 5′-diphosphate-glucuronosyltransferases, sulfotransferases, glutathione S-transferases, and ATP-binding cassette transporters.1,2) They form heterodimers with retinoid X receptor (RXR) and bind to common regulatory sequences in the promoter regions of target genes. PXR and CAR were first identified as a regulator of CYP3A and CYP2B genes, respectively, but subsequent studies have demonstrated their crosstalk (i.e., CYP3A regulation by CAR and CYP2B regulation by PXR). Moreover, nuclear receptors identified as receptors responsive to endobiotics such as hormones, vitamins, and lipids (e.g., liver X receptors or LXRs, peroxisome proliferator-activated receptors or PPARs, vitamin D receptor or VDR) have been reported involved in gene regulation of drug-metabolizing enzymes and transporters.3) Our group found considerable crosstalk between multiple nuclear receptors and drug disposition-related genes.3–8) In addition to drug metabolism and disposition, PXR and CAR play significant roles in energy homeostasis in the liver to adapt animals to xenobiotic stress.9–18) Moreover, their functions in immunological and inflammatory responses in the liver and extrahepatic tissues have been reported.19–24) Thus PXR and CAR work in not only xenobiotic disposition but also diverse physiological and pathophysiological systems as xenobiotic sensors.2,25)

It is well known that there are species differences in the activators of these xenobiotic-responsive nuclear receptors based on structural differences in their ligand-binding domains (LBDs).1,2,26) For example, the anti-tubercular drug rifampicin binds to and activates human PXR (hPXR) but does not activate rodent PXR whereas pregnenolone 16α-carbonitrile (PCN) activates rodent PXR but not hPXR. Thus rifampicin exhibits its PXR-mediated effects in humans but not rodents and PCN vice versa. Despite such species differences in their activators, the fundamental functions of PXR and CAR seem analogous to each other. Once activated, they induce expression of similar sets of genes, such as those associated with drug metabolism and disposition as described above, to protect the body against harmful xenobiots both in humans and rodents.1,2) For other relatively new functions of PXR and CAR, such as energy metabolism, it remains uncertain whether there is species difference between rodents and humans since most studies were carried out with animal models and little has been tested in clinical studies. It should be noted that there is a clear species difference in CAR-mediated hepatocyte proliferation and chemical carcinogenesis as described below in detail.

Among the diverse roles of PXR and CAR, we are in-
terested in those in hepatocyte proliferation and chemical hepatocarcinogenesis. As described below in detail, the CAR activator phenobarbital is one of the most well-known liver tumor promoters \(^{27,28}\) and it has been demonstrated that CAR is necessary for phenobarbital-induced liver tumor formation in mice. \(^{29,30}\) There are several reports on the mechanism for CAR-mediated hepatocyte proliferation, but some details remain unclear (Fig. 1). Meanwhile, little is known about the association of PXR with liver tumor formation despite that PXR and CAR share various characteristics in terms of structure, activators, and functions (Fig. 1). In this manuscript, I will review the background and our recent results on the role of PXR in hepatocyte proliferation and the mechanism for CAR-mediated hepatocyte proliferation. \(^{31–34}\)

2. PXR AND HEPATOCYTE PROLIFERATION/HEPATOCARCINOGENSES

It is well known that PXR activation does not induce hepatocyte proliferation in rodents. \(^{35}\) Although they increase liver size, it is considered that the liver enlargement largely results from hepatocyte hypertrophy associated with CYP induction. \(^{36,37}\) Staudinger \textit{et al.} \(^{38}\) reported that intraperitoneal injection of mice with PCN, a representative rodent PXR ligand, at a relatively high dose (once daily at 400 mg/kg for 4 consecutive days), increased the number of proliferating cell nuclear antigen (PCNA)-positive nuclei in the liver. For other cell and tissue types, there are some controversial reports on the association between PXR and cell growth. PXR upregulated protein levels of cyclin-dependent kinase (CDK) inhibitor p21, suppressing the growth of colon cancer cells, \(^{39}\) and PXR overexpression suppressed growth of neuroblastoma cells, \(^{40}\) suggesting that PXR has an anti-proliferating function. In contrast, other reports indicate that PXR is associated with cancer cell growth. In esophageal squamous carcinoma and breast, endometrial, and prostate cancer cells, PXR expression levels were higher than those in normal tissues. \(^{41–44}\) In addition, PXR activation was shown to induce proliferation of colon cancer cells \textit{in vitro} and in tumor-bearing mice. \(^{45}\)

Since CAR and PXR belong to the same subfamily (NR1I) of the nuclear receptor superfamily, their functions are assumed similar. Indeed, PXR and CAR coordinately regulate expression of genes associated with drug metabolism and disposition and protect the body from the xenobiotic exposure. In contrast, the role of PXR in cell cycle regulation and carcinogenesis remains unclear despite that CAR has long been known as one of the key factors for chemical hepatocarcinogenesis. We thus started experiments to elucidate clues to this question on PXR and hepatocarcinogenesis.

2.1. Stimulation of Xenobiotic-Induced Hepatocyte Proliferation

We first performed very simple experiments to investigate the influence of PXR activation on hepatocyte proliferation. Mice were intraperitoneally treated with PCN at a dose of 100 mg/kg, which is high enough to activate PXR \textit{in vivo}, in combination with or without the potent mouse CAR ligand, 1,4-bis-[2-(3,5-dichloropyridyloxy)]-benzene (TCPOBOP). The reason that we treated mice with PCN and TCPOBOP simultaneously was because we hypoth-
esized that PXR could antagonize CAR-dependent hepatocyte proliferation based on a report showing that PXR inhibited tumor cell growth.\(^{39}\) This hypothesis seemed too easy and we soon found that this was not the case but the results were very surprising and interesting (see below), which drove us to start a series of experiments on the involvement of PXR in hepatocyte proliferation.\(^{31,32,34}\)

Single treatment of mice with TCPOBOP increased the number of proliferation marker Ki-67-positive hepatocytes 48 h after the treatment whereas PCN did not, as expected. These findings were confirmed by determining mRNA levels of cell cycle-related genes such as Ccna2, Cenbl, Mcm2, or Mki67. Unexpectedly, co-treatment with PCN and TCPOBOP increased the number of proliferating hepatocytes (approx. 10-fold) more than TCPOBOP single treatment (approx. 3-fold), judged from Ki-67 immunohistochemistry. When we increased the number of proliferating hepatocytes (approx. Mki67\(^{Pxr}\)) liver regeneration after partial hepatectomy was delayed in \(^{31}\) The results imply that PXR increase in the hepatic mRNA levels of Cyp2b10, which is a representative CAR target gene.\(^{30}\) The results imply that PXR does not simply enhance the transcriptional activity of CAR but exhibits the stimulating effect on hepatocyte proliferation through a different mechanism associated with cell cycle regulation. To corroborate this hypothesis, we treated mice with Wy-14643, a PPAR\(^{α}\) ligand, as another proliferative stimulus via a different mechanism and investigated the influence of PXR co-activation. As we expected, PCN co-treatment also enhanced Wy-14643-induced hepatocyte proliferation and PCN co-treatment did not affect the increase in the hepatic mRNA levels of Cyp4a10, a representative PPAR\(^{α}\) target gene.\(^{31}\)

Since PXR is activated by a wide range of chemicals,\(^{26,47}\) we sought to know whether chemical compounds other than PCN could exhibit cell proliferation-stimulating effects through PXR activation. Since there is little known mouse PXR (mPXR) ligand except PCN, we screened chemical compounds including industrial chemicals, pesticides, and food additives and related compounds and found that imazalil (also known as enilconazole) activates mPXR to enhance hepatocyte proliferation induced by the CAR ligand TCPOBOP in mouse livers.\(^{34,48}\) These results suggest that the stimulating effect of PXR on xenobiotic-induced hepatocyte proliferation is a characteristic common to various types of PXR ligands.

### 2.2. Acceleration of Growth Factor-Associated Hepatocyte Proliferation

Hepatocyte proliferation is induced by not only xenobiotic exposure but also liver injury or partial hepatectomy to recover normal size and functions of the liver. It has been reported that treatment of rats with PCN after partial hepatectomy enhanced the regeneration.\(^{49}\) Consistently, liver regeneration after partial hepatectomy was delayed in Pxr-null mice.\(^{50}\) Taken together with our findings mentioned above, we raised a possibility that PXR could also stimulate liver injury-induced, \textit{i.e.}, growth factor/cytokine-induced, hepatocyte proliferation. To investigate this possibility, we carried out a series of experiments using \textit{in vitro} and \textit{in vivo} models.\(^{31,32}\)

We first used immortalized mouse normal hepatocytes, AML12 cells, as an \textit{in vitro} model.\(^{32}\) When AML12 cells are cultured in the absence of serum for 48 h, they become synchronized at the G0 phase, and we are able to make the cells enter the cell cycle by cultivating them in medium containing serum. These characteristics are suitable for investigating the cell cycle regulation by PXR. Since PXR expression levels in AML12 cells are relatively low compared with those in mouse livers, we infected AML12 cells with adenovirus expressing mPXR and treated with PCN in medium with or without serum, and analyzed cell cycle distributions of the cells by flow cytometry. The results demonstrated that PCN treatment in combination with mPXR expression augmented the serum-mediated decrease in the number of cells at the G0/G1 phase and increase in those at the S and G2/M phases. Consistently, PXR activation increased PCNA protein levels and mRNA levels of cell cycle-related genes, and accelerated cell growth. This PXR-induced growth acceleration of AML12 cells was also observed with epidermal growth factor (EGF) instead of serum.

As an \textit{in vivo} model, we chose a carbon tetrachloride (CCL\(_4\))-induced acute liver injury model. It is reported that hepatocyte proliferation starts around 24 h after CCL\(_4\) treatment\(^{51}\) and the proliferating hepatocytes are at the G1, S, or G2 phase and scarcely at the M phase 48 h after treatment.\(^{52}\) Mice were first treated intraperitoneally with CCL\(_4\) and 24 h later with PCN, and hepatocyte proliferation was investigated 24 h after PCN treatment. As expected, PCN co-treatment enhanced CCL\(_4\)-induced increase in the number of Ki-67-positive hepatocytes as well as hepatic mRNA levels of cell cycle-related genes, and these enhancements were not observed in Pxr-null mice, suggesting that the enhancement is PXR-dependent.\(^{32}\)

To confirm these \textit{in vivo} enhancing effects of PXR, we investigated the influence of PXR activation on hepatocyte proliferation induced by fibroblast growth factor 19 (FGF19), which is reported to induce hepatocyte proliferation in rodents.\(^{53,54}\) Mice were treated subcutaneously with recombinant FGF19 with or without PCN co-treatment for 3 d and hepatocyte proliferation was investigated. The results were similar to those obtained with the CCL\(_4\) model. PCN co-treatment enhanced FGF19-induced hepatocyte proliferation judged by Ki-67 immunohistochemistry and qRT-PCR.\(^{55}\) More recently, Jiang \textit{et al.}\(^{55}\) reported that liver regeneration after two thirds partial hepatectomy in mice was augmented by PCN treatment with the increased number of Ki-67-positive hepatocytes at earlier time points.

Based on the results obtained from our \textit{in vitro} and \textit{in vivo} studies\(^{31,32}\) and other groups’ \textit{in vitro} studies,\(^{39,50,55}\) it is strongly suggested that PXR activation stimulates growth factor- and/or cytokine-induced proliferation of murine hepatocytes. Taken together with the results using CAR and PPAR\(^{α}\) activators, we proposed that PXR has a novel and unique function in cell cycle regulation: PXR enhances hepatocyte proliferation induced by multiple types of stimuli including xenobiotics and growth factors without obvious ability to induce hepatocyte proliferation by itself (Fig. 2).

### 2.3. Mechanism for Stimulation of Hepatocyte Prolif-
eration The results obtained in in vitro and in vivo studies raised a possibility that PXR activation could affect a common machinery of cell cycle. Since mature hepatocytes are usually at quiescent states (the G0 phase) and the cell cycle progression from the G0 to G1 and from the G1 to S phases is tightly regulated by cell cycle suppressor genes, we first investigated the influence of PXR activation on the expression of these genes.\textsuperscript{32} We found that PCN treatment decreased expression of several cell cycle suppressor genes, including \textit{Cdkn1b}, \textit{Rbl2}, \textit{Cdkn1a}, \textit{Cdkn1c}, \textit{Cdkn2a}, and \textit{Cdkn2b}, 24 h after treatment in wild-type but not PXR-deficient mice. These suppressions were also observed in AML12 cells. Given that the expression levels of these cell cycle suppressor genes are associated with the sensitivity of cells to proliferating stimuli,\textsuperscript{56,57} PXR activation might accelerate cell cycle progression by down-regulating expression of cell cycle suppressor genes, which is consistent with the observation that PXR activation enhances hepatocyte proliferation induced by diverse types of stimuli, including CAR activators, PPAR\textsubscript{\alpha} activators, growth factors, and liver injury.

In agreement with this idea, we demonstrated that PCN treatment shifted the cell cycle of mouse hepatocytes from the G0 to G1 phase in vivo, using flow cytometry.\textsuperscript{31} Moreover, chronological analysis of hepatocyte proliferation in CCl\textsubscript{4}-induced liver injury model demonstrated acceleration of cell cycle progression of mouse hepatocytes in vivo after PCN treatment.\textsuperscript{32} The usual peak time of the number of Ki-67-positive nuclei and the increase in \textit{Ccnb2} and \textit{Ccnb1} mRNA levels was at around 72 h after CCl\textsubscript{4} administration in the model. Interestingly, co-treatment with PCN shifted the peak to earlier time points. Indeed, the number of M-phase hepatocytes at 48 h after CCl\textsubscript{4} treatment was significantly increased by PCN treatment in wild-type but not \textit{Pxr}-null mice. These results corroborate the idea that PXR is able to accelerate cell cycle progression of proliferating hepatocytes on exposure to proliferating stimuli including xenobiotics and liver injury.

It is known that FOXO family transcription factors commonly regulate expression of cell cycle suppressor genes.\textsuperscript{58} We then investigated the crosstalk between PXR and FOXO proteins.\textsuperscript{32} We found that PCN treatment reduced hepatic expression of \textit{Pck1}, a FOXO1 target gene, and \textit{Mxi1} and \textit{Bim}, FOXO3 target genes in mice, in a PXR-dependent manner. Moreover, FOXO3-dependent expression of a luciferase reporter gene under the control by the mouse \textit{Rbl2} promoter, which includes two FOXO binding sites, was suppressed by ligand-activated PXR, and FOXO3-mediated expression of \textit{Rbl2} and \textit{Mxi1} in mouse primary hepatocytes was decreased by PCN treatment. Finally, we demonstrated physical interaction between PXR and FOXO3 in AML12 cells by co-immunoprecipitation assay.

Based on these findings, the proposed mechanism for PXR action on cell cycle regulation is depicted in Fig. 3. The interaction of PXR with FOXO proteins to downregulate expression of cell cycle suppressor genes might be a key event for PXR-mediated stimulation and acceleration of hepatocyte proliferation. Since we have been studying only using mouse systems, its human relevance needs to be explored in future studies.

\subsection*{2.4. PXR and Hepatocarcinogenesis}

Chemical carcinogenesis is generally divided into multiple stages including initiation, promotion, and progression.\textsuperscript{59} At the initiation stage, DNA damage leading to mutations or other types of alteration of genes is induced by exposure to certain chemical compounds (i.e., carcinogenic compounds) such as dimethylnitrosamine, ethylnitrosourea, nitrogen mustard, heterocyclic amines, aryl hydrocarbons, and metal-related compounds. DNA damage may produce “initiated cells,” in which the functions of oncogenes and/or tumor suppressor genes are altered. In the promotion stages, the “initiated” or preneoplastic...
cells are actively proliferated. Chemical compounds can also modify this proliferation process. For example, CAR activators such as phenobarbital and PPARα activators such as hypolipidemic fibrates induce hepatocyte proliferation at least in rodents and thus they are known as “liver tumor promoters.” Okadaic acid, a phosphatase inhibitor, and 12-O-tetradecanoylphorbol 13-acetate (or phorbol 12-myristate 13-acetate) induce proliferation of epidermal cells and skin cancer through altering cell signaling; thus they are also “tumor promoters.” In contrast, some food-derived chemopreventive agents may prevent or weaken this promotion stage. Finally, in the progression stage, the initiated and proliferated cells undergo neoplastic transformation and phenotypic changes. The tumor size drastically increases in this stage and the cells become more invasive and metastatic with multiple mutations.

In this multistage carcinoogenesis model, both CAR and PPARα activators act as “promoters” because they are able to induce hepatocyte proliferation but unable directly to induce DNA damages. In contrast, PXR activators are not promoters since our results indicate that PXR activation itself does not induce cell proliferation at least in mouse livers. Instead, PXR stimulates hepatocyte proliferation mediated by xenobiotics with CAR and PPARα as well as growth factors. Although it remains to be elucidated whether PXR activation increases the incidence of liver tumor induced by CAR or PPARα activators or other stimuli, if this is the case, PXR activators might be novel types of chemical compounds that act on multistage carcinoogenesis model (Fig. 4). They might work as “enhancers,” which enhance the actions of promotors without showing promoting effects by themselves. We are currently working on long-term carcinoogenesis experiments to clarify the role of PXR in chemical hepatocarcinoogenesis.

3. CAR AND HEPATOCYTE PROLIFERATION/HEPATOCA RINOCARCINOGENESIS

The regulation mechanism of CAR activity as a transcription factor differs from other typical nuclear receptors in some points. First of all, CAR is constitutively active, and its transcriptional activity is regulated by its cellular localization. Without any stimuli, the receptor is largely retained in the cytoplasm, but on exposure to xenobiotics, including pharmaceutical drugs and environmental pollutants, CAR is translocated into the nucleus to promote transcription of its target genes. Second, CAR is activated not only by ligands that bind directly to CAR but also by chemical compounds that do not bind to CAR. The latter types of CAR activators induce CAR nuclear translocation by cellular phosphorylation/de-phosphorylation signals involving EGF receptor, protein phosphatases, and some adaptor proteins such as RACK1. Phenobarbital, one of the well-known CAR activators, is classified as the latter indirect activator.

Phenobarbital has long been known as a nongenotoxic hepatocarcinogen or liver tumor promoter, and prolonged exposure to phenobarbital induces hepatocyte proliferation and liver tumor formation at least in rodents, and this tumor formation has been demonstrated CAR-dependent using Car-null mice. However, the results of several epidemiological studies indicate that phenobarbital-induced liver tumor is not relevant to humans, although the molecular basis on this species difference remains unknown.

To understand the species differences in CAR-induced hepatocarcinogenesis, elucidation of the molecular mechanism for CAR-dependent hepatocyte proliferation in rodents is essential. Previous reports show that CAR activation increases expression levels of Myc, an oncogene, and its target gene Foxm1. Also, mRNA levels of Gadd45b, encoding a modulator of p53, and Ccnd1, which induces resting hepatocytes to enter the G1/S phases, were increased after TCPBOB treatment in mouse livers. These results provide useful information on the mechanism, further studies are necessary; especially, the target gene(s) or protein(s) of CAR as a first step to initiate cell proliferation is of great interest.

3.1. In Vitro Model for CAR-Dependent Hepatocyte Proliferation

Model animals such as knockout mice lacking key factor(s) for cell proliferation are useful for investigation of CAR-mediated hepatocyte proliferation and hepatocarcino genesis. Meanwhile, cell-based in vitro systems are often used to reveal molecular mechanisms in detail. One of the reasons that the mechanism for the CAR-dependent hepatocyte proliferation remains unclear is the lack of an appropriate in vitro system(s) to reproduce CAR-dependent cell proliferation. Unfortunately, there is no cell line reported to express CAR as strongly as in the liver or primary hepatocytes. We thus aimed to construct such an in vitro system.

Primary hepatocytes could be used for such a study since they retain various liver-related functions, but we found that expression levels of CAR in mouse hepatocytes were drasti-
cell activation reduced within 24 h after plating. We thus expressed mouse CAR (mCAR) using an adenovirus vector and tested its influence on proliferation of mouse primary hepatocytes and AML12 cells.30 With mCAR expression, mouse primary hepatocytes proliferated in a multiplicity of infection (MOI)-dependent manner. The number of hepatocytes expressing mCAR and treated with TCPOBOP became more than 2-fold 5 d after adenovirus infection compared with control cells. In cells with activated CAR, mRNA levels of a CAR target gene (i.e., Cyp2c55) as well as those associated with cell proliferation (Ccna2, Ccnb1, Nek2, Mcm2, and Foxm1) were increased, supporting the transcriptional activation of CAR and cell cycle progression in this system. This mCAR-dependent cell proliferation was also observed with AML12 cells.

As described above, we found that PXR activation enhances CAR-mediated hepatocyte proliferation in mice. We thus tested whether this enhancement was retained in the in vitro system with primary hepatocytes. As expected, adenosine expression of mPXR in combination with PCN treatment did not affect the cell number, but co-expression of mPXR and mCAR with respective ligand treatment increased the cell number more markedly than single expression of mCAR.30

These results indicate that we are able to monitor and investigate CAR-induced hepatocyte proliferation using the in vitro system established.

3.2. Role of YAP in CAR-Mediated Hepatocyte Proliferation

The Hippo pathway is a signal transduction pathway that consists of multiple kinases including MST1/2 and LATS1/2, and controls cell and organ size.64 Yes-associated protein (YAP) is known as an effector protein of the pathway and works as a transcriptional cofactor, playing a vital role in liver hypertrophy and carcinogenesis.65 In normal livers, YAP is phosphorylated by Hippo pathway kinases in the cytoplasm and actively degraded.66,67 It is well known that in liver cancer and other carcinomas the Hippo pathway is dysregulated and active YAP is accumulated in the nucleus,68,69 where it modulates transcription by several transcription factors such as TEA domain family members (TEADs), PPARγ, SMADs, and p73.69 Kowalik et al.70 recently reported that treatment with the mCAR agonist TCPOBOP increased nuclear YAP levels in mouse livers, although the causal relation between CAR activation, YAP activation, and hepatocyte proliferation remains unclear. With this background, we investigated the role of YAP using in vitro studies and the established in vitro system.

We first confirmed CAR activation-induced YAP activation in vivo. TCPOBOP treatment of mice increased hepatic mRNA levels of the CAR target genes Cyp2b10 and Cyp2c55 and cell proliferation marker genes, such as Ccna2, Ccnb1, Mcm2, and Foxm1, as well as the number of Ki-67-positive hepatocytes in the liver. More importantly, the treatment induced accumulation of nuclear YAP, which was detected by Western blot analysis of liver nuclear extracts and immunohistochemistry. Consistently with this, mRNA level of YAP target genes, Birc5, Myc, and Ankrd1, were also increased in mouse liver by TCPOBOP treatment. Co-treatment with verteporfin, which is reported to inhibit the interaction between YAP and TEAD,71 prevented the TCPOBOP-induced hepatomegaly and tended to reduce YAP target gene mRNA levels as well as those of Ccna2, Ccnb1, and Mcm2. Taken together with the report by Kowalik et al.,70 our results suggest that the YAP/TEAD system plays a role in CAR-dependent hepatocyte proliferation at least in mice.

Functional requirement of YAP in CAR-dependent hepatocyte proliferation was next investigated using the in vitro system established.33 The small interfering RNA (siRNA)-mediated knockdown of YAP in AML12 cells almost completely blocked or drastically attenuated the mCAR/TCPOBOP-dependent increase in mRNA levels of cell cycle-related genes as well as YAP target genes. Moreover, mCAR-dependent proliferation of AML12 cells was inhibited by YAP knockdown. Consistently, verteporfin treatment blocked the CAR-dependent growth of AML12 cells and increases in YAP-target gene expression. These results of functional and pharmacological inhibition of YAP strongly suggest that YAP is indispensable for CAR-dependent hepatocyte proliferation in mice.

The crosstalk between CAR and YAP in gene transcription was investigated using reporter assays with TEAD-responsive reporter plasmid and dominant active form of mouse YAP (mYAP-5SA), in which all five putative phosphorylation sites72,73 are mutated to alanine.33 In AML12 cells, mYAP-5SA activated the reporter gene expression in a dose-dependent manner and mCAR activation enhanced this YAP-TEAD-mediated gene expression. Interestingly, mYAP-5SA expression enhanced CAR-dependent gene expression as well in reporter assays using a reporter plasmid containing CAR-responsive elements. These results suggest the presence of functional crosstalk between CAR and YAP/TEAD in gene transcription (Fig. 5).

While phenobarbital is known as a liver tumor promoter, its prolonged exposure induces liver tumor formation in rodents without treatment with any initiating compounds.27,28 Since phenobarbital efficiently induces multiple forms of CYPs through CAR activation, reactive oxygen species produced under CYP-induced condition may cause more DNA damage. Thus CAR activation induces liver tumor formation as a result of DNA damage (i.e., initiation) via CYP induction and hepatocyte proliferation (promotion) via YAP activation (Fig. 5).

3.3. Human Relevance of CAR-Mediated Hepatocyte Proliferation

Several studies of CAR-mediated liver tumor formation demonstrate that replicative DNA synthesis and hepatocyte proliferation are key events,34 although precise mechanisms on how CAR activation induces cell cycle progression of resting mature hepatocytes remain unclear. Replicative DNA synthesis and hepatocyte proliferation are also considered key events causing species differences in CAR-mediated liver tumor formation between rodents and humans.34 Phenobarbital, a well-known liver tumor promoter in rodents, activates CAR in mice, rats, and humans, but induces DNA synthesis and hepatocyte proliferation only in rodents. For example, Yamada’s group15,76 demonstrated that hepatocyte growth factor increased DNA synthesis (BrdU labeling) in rat and human hepatocytes in vitro but phenobarbital did only in rat hepatocytes whereas it increased CYP2B6 and CYP2B1 mRNA levels in human and rat hepatocytes, respectively.

To confirm these results, studies using transgenic mice having human CAR have been conducted and contradictory results were reported. At first, Huang et al.30 showed that phenobarbital treatment induced murine hepatocyte proliferation and showed anti-apoptotic effects using CAR-knockout mice carrying human CAR, which is inconsistent with the idea that phenobarbital does not induce liver tumor formation in...
humans.30) Studies using transgenic mice, in which mouse CAR and PXR are replaced with human CAR and PXR, also demonstrated transient induction of DNA replication after phenobarbital treatment.77) In contrast, another study with the same CAR/PXR-humanized mice demonstrated that phenobarbital treatment caused CYP2B induction, liver enlargement, and centrilobular hepatocyte hypertrophy but not DNA synthesis (BrdU labeling).78) Moreover, in a two-stage (initiation and promotion) carcinogenesis study, phenobarbital treatment promoted diethylnitrosamine-initiated liver tumor formation not only in wild-type but also in CAR/PXR-humanized mice although the incidence was lower in the humanized mice than wild-type mice.79) Thus no clear conclusion has been drawn, but it should be noted that the amount and treatment period of phenobarbital is different among the studies and that human CAR in mouse hepatocytes might act differently from that in human hepatocytes. More recently, studies using chimeric mice with humanized livers have been conducted using phenobarbital and other CAR activators.76,80) Their results indicate that CAR activation does not induce replicative DNA synthesis in human hepatocytes, supporting our current understanding on the species differences.

We have demonstrated that a functional interaction with YAP is critical for CAR-mediated mouse hepatocyte proliferation. Therefore studies focusing on species differences in the CAR-YAP interaction between rodents and humans might provide clues to the question on the human relevance of CAR-mediated liver tumor formation.

4. CONCLUSION AND FUTURE PERSPECTIVES

Recent studies have demonstrated diverse roles of PXR and CAR beyond drug metabolism and disposition. As expected from the similarity in their structures, the functions of PXR and CAR are also similar in most cases. However, in terms of chemical carcinogenesis, there remain several open questions. Our group has been working on the role of PXR in hepatocyte proliferation and chemical hepatocarcinogenesis and the mechanisms for CAR-dependent hepatocyte proliferation.

As reviewed in this article, we found a unique function of PXR. Unlike CAR, PXR does not induce hepatocyte proliferation in mice but it accelerates the cell cycle progression of proliferating murine hepatocytes exposed to various types of stimuli through reducing the FOXO-dependent expression of cell cycle suppressor genes (Figs. 2, 3). Based on these findings, I have raised a possibility that PXR activators work as “enhancers,” which enhance the action of promoters in multistage carcinogenesis model (Fig. 4). This possibility should be clarified in future studies.

We have also demonstrated in mice that YAP is crucial for CAR-dependent hepatocyte proliferation and CAR enhances YAP/TEAD-dependent gene expression using the newly developed in vitro system of CAR-mediated hepatocyte proliferation (Fig. 5). I believe that these findings will give us new insights into the role of PXR and CAR in chemical carcinogenesis and help us understand the mode of action of carcinogenic compounds and its human relevance.

In both systems, protein–protein interactions, i.e., PXR-FOXO and CAR-YAP/TEAD, are fundamental to the actions of PXR and CAR. The classical mechanism for the actions of nuclear receptors is binding to the promoter regions of their target genes via DNA-binding domains (DBDs). Since the homology of DBDs is much higher than those of LBDs in the case of PXR and CAR83) and the promoter regions of essential genes such as cell cycle suppressor genes and the Hippo pathway-related genes should be conserved among species, the classical pathway would barely cause species differences in the actions of PXR and CAR. Indeed, these receptors regulate expression of drug-metabolizing enzymes in rodents and humans through this classical mechanism. In contrast, it is well known that structural differences in the LBDs cause species differences in chemical activation. In addition to ligand binding, low homology of the LBD structures may cause species differences in protein–protein interactions through LBDs. Until now, we have been working mainly with mouse models and precise mechanisms at the molecular level including the interfaces of the protein–protein interactions remain to be elucidated. Future studies will help us to understand species dif-
ferences in the actions of PXR and CAR in hepatocyte proliferation and chemical carcinogenesis at molecular levels, which would be useful for drug discovery and development as well as risk assessment of chemical compounds such as residual pesticides, food contaminants, and environmental pollutants.

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