Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Deregulation of Growth Factor, Circadian Clock, and Cell Cycle Signaling in Regenerating Hepatocyte RXR α -Deficient Mouse Livers

Xiaoxia Yang, Minglei Guo,
and Yu-Jui Yvonne Wan

*From the Department of Pharmacology, Toxicology, and
Therapeutics, The University of Kansas Medical Center,
Kansas City, Kansas*

Activation of the nuclear receptors constitutive androstane receptor, pregnane X receptor, and peroxisome proliferator-activated receptor α results in hepatomegaly, and these nuclear receptors are implicated in the regulation of liver regeneration. Retinoid X receptor (RXR) α is an essential partner of these nuclear receptors. Therefore, we studied the role of hepatocyte RXR α in liver regeneration using partial hepatectomy model. The results showed that hepatocyte RXR α deficiency caused an approximately 20-hour delay in hepatocyte proliferation after partial hepatectomy. Several pathways, including growth factors and the circadian cell cycle, were impaired due to hepatocyte RXR α deficiency. In addition, the expression patterns of hepatocyte growth factor, fibroblast growth factor 2, platelet-derived growth factor, and transforming growth factor α were altered due to lack of RXR α . Furthermore, the peroxisome proliferator-activated receptor α /brain and muscle Arnt-like protein 1/Rev-erba/P21 pathway was compromised, and Cry1/Cry2 and Wee1/Per1 expression was deregulated in regenerating RXR α -null livers. Accordingly, the expression and regulation of cyclin D1/Cyclin-dependent Kinase (Cdk)4, cyclin E1/Cdk2, cyclin A2/Cdk2, and cyclin B1/Cdk1 after partial hepatectomy were altered in regenerating RXR α -null livers. Hepatocyte RXR α deficiency also affected the basal, as well as regeneration-induced cyclin E1 expression levels. Activation of RXR α by retinoic acids increased the cyclin E1 promoter activity indicating retinoic acid-mediated signaling positively controls *cyclin E1* gene expression. As many of these observed changes were not documented in the regenerating livers of other nuclear receptor knockout mice, these observed ef-

fects may be hepatocyte RXR α specific. (Am J Pathol 2010, 176:733–743; DOI: 10.2353/ajpath.2010.090524)

Liver regeneration induced by two-thirds partial hepatectomy (PH) proceeds along a sequence of distinctive phases and results in a precise reconstitution of the lost liver mass.¹ Many nuclear receptor-mediated signals have been implicated in the proliferative response in the regenerating liver. Farnesoid X receptor [FXR (also known as Nr1h4)]-dependent bile acid homeostatic signaling is associated with proliferative events leading to the regeneration of the liver. FXR knockout mice show defective regeneration.² Peroxisome proliferator-activated receptor α (PPAR α)-mediated Ras and RhoA membrane association plays a key role in early DNA synthesis and cell proliferation.³ Disruption of PPAR α -mediated lipid signaling pathway transiently impairs the initiation of liver regeneration.⁴ However, other studies show that PPAR α deficiency does not affect liver regeneration following PH.^{5,6} This discrepancy might be in part due to differences in the genetic backgrounds of the mice used.⁵ Pregnane X receptor (PXR, also known as Nr1i2) mediates hepatocyte proliferation during both early and late stages of liver regeneration. Lack of PXR impairs normal progression of liver regeneration.⁷ Delayed hepatocyte proliferation is also noted in constitutive androstane receptor (also known as Nr1i3) knockout mice.²

Retinoid X receptor α (RXR α) is the most abundant RXR among the three RXR isoforms (α , β , and γ).⁸ RXR α forms dimers with FXR, PPAR α , constitutive androstane receptor, and PXR and regulates gene transcription.⁹

Supported by National Institutes of Health grants CA53596 and COBRE P20 RR021940.

Accepted for publication September 29, 2009.

Current address of M.G.: Department of Basic Medical Science, School of Medicine, University of Missouri-Kansas City, 2411 Holmes, Kansas City, MO, 64108.

Address reprint requests to Yu-Jui Yvonne Wan, Ph.D., Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, 4059 Kansas Life Sciences Innovation Center, 3901 Rainbow Blvd, Kansas City, KS 66160. E-mail: ywan@kumc.edu.

Table 1. Nucleotide Sequences of TagMan Probes (with 5'-FAM and 3'-BHQ1 Modifications) and Primers for the Studied Genes

Genes	Probe sequence	Forward primer	Reverse primer
<i>Acox1</i>	5'-TGGGACCACAAGCCTCTGCCA-3'	5'-TTTGTGTGTCCTATCCGTGAGA-3'	5'-GCCGATATCCCCAACAGTGA-3'
<i>Actb</i> (β -actin)	5'-CCACACCCGCCACAGTTGCC-3'	5'-CTTCTTTGAGCTCCTTCGTG-3'	5'-CGACCAGCCGACGATATC-3'
<i>Albumin</i>	5'-CAAAATGCCATTCTAGTTTCGCTACACCCAG-3'	5'-ACGAGAAGCTTGAGAAATATGGA-3'	5'-GCCCACTCTTCCTAGGTTTCTTG-3'
<i>Arntl</i> (<i>Bmal1</i>)	5'-TAGGTGCAATGATCGCGGAGGA-3'	5'-CAGAGCCGAGCAGGAAA-3'	5'-AAGCGATGACCTCTTATCC-3'
<i>CDK2</i>	5'-CTTTCTGCCATTCTCACCGTGCCTTCA-3'	5'-TCCCCTCATCAAGAGCTATCTGTT-3'	5'-TCTGCATTGATAAGCAGTTCTG-3'
<i>Cyclin A2</i>	5'-AAACAGAGTGTGAAGATGCCCTGGCTTTT-3'	5'-GAAGAGGCCAGCTGAGCTTAAA-3'	5'-CGGGTAAAGAGACAGCTGCAT-3'
<i>Cyclin B1</i>	5'-TGAACCTTCAGCCTGGGTGCGCC-3'	5'-GACAGATGGAGATGAAGATTCTCAGA-3'	5'-ACGTCAACCTCTCCGACTTTAGA-3'
<i>Fabp1</i>	5'-TCACCATCACCTATGGACCCAAAGTGG-3'	5'-TGCATGAAGGGAAGAAAATCAAA-3'	5'-CCCCAGGGTGAACCTATT-3'
<i>Fgf2</i>	5'-CGCGAGAAGAGCCACCCACACG-3'	5'-GACGGCCGCTGGAT-3'	5'-CTCTCTTCTGCTTGGAGTTGTAGTTTG-3'
<i>Hgf</i>	5'-TGGCATCAATGCCAGCCTTGGA-3'	5'-AAGGGACGGTATCCATCACTAAGA-3'	5'-GGGTTGAAGGCACGACAT-3'
<i>Nr1d1</i> (<i>Rev-erbα</i>)	5'-CCCATGGACGACGCGCC-3'	5'-GCAAGGCAACACCAAGAATGT-3'	5'-CTTCCCAGATCTCTGACAGT-3'
<i>P21</i>	5'-ACATTCAGAGCCACAGGCACCATGTC-3'	5'-GCAGATCCACAGCGATATCCA-3'	5'-GGTCGGACATCACCAGGATT-3'
<i>PPARα</i>	5'-TCTGTCCGGATGTCACACAATGCAATTC-3'	5'-GATTCAGAAGAAGAACCGGAACA-3'	5'-TGCTTTTTCAGATCTTGGCATTTC-3'
<i>Tgfa</i>	5'-CCTGGTGGTGGTCTCCATTGTGGC-3'	5'-CCAGAAGAAGCAAGCCATCACT-3'	5'-GTGGATCAGCACACAGGTGATAA-3'
<i>Wee1</i>	5'-TTTCCAAGCGCAGTTATTGAT-3'	5'-GCCGCACACTCCCAAGAG-3'	5'-AGAACTACCCCGAGTTTAAACAGA-3'

Lack of RXR α impairs activation of its partners and subsequently affects the downstream cascades. In addition, RXR α forms homodimers and heterodimers with retinoic acid receptors. RXR α is essential in the transduction of a retinoid signal required for myocardial development and ocular morphogenesis, suggesting that RXR α is critical for cell proliferation and differentiation.¹⁰ Lack of RXR α results in a transient delay in liver growth during embryonic development.¹¹ In the current study, we investigated the modulation of liver regeneration by RXR α and the underlying mechanisms involved.

Materials and Methods

Animals and PH

Age and genetic background (mixed background of C57/Bl/6, 129/SvEvTac, and DBA-2) matched wild-type and hepatocyte RXR α -deficient mice (3 to 4 months old) were used for partial hepatectomy. Hepatocyte RXR α -deficient mice were generated by deletion of exon 4 of the *RXR α* gene using albumin-cre/lox-mediated recombination.¹² The control mice had RXR α loxP/loxP (cre negative) genotype. The mice were housed with a 12:12 hour light/dark cycle. Standard two-thirds liver resection was performed at 10:00 AM according to the procedure described by others.¹³ Sham-operated mice were included as controls. Mice were sacrificed at the indicated time points and liver samples were immediately excised, snapped frozen in liquid nitrogen, and stored at -80°C until analysis. Liver samples were also fixed in 10% buffered formalin for immunohistochemistry. All of the animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Ki-67 Immunostaining

Ki-67 immunostaining was performed using primary Ki-67 antibody (NeoMarkers, Fremont, CA) according to the manufacturer's instruction to monitor hepatocyte proliferation. The number of Ki-67-labeled nuclei was determined by counting the Ki-67-positive hepatocytes in at least five microscope fields ($\times 20$) for each sample with $n = 3$ to 6 mice/time point.

Quantitation of mRNA by Real-Time Polymerase Chain Reaction

Hepatic RNA isolated using TRIzol (Invitrogen, Carlsbad, CA) was used for reverse transcription followed by amplification of cDNA using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). Primers and probes were designed using Primer Express 2.0 according to the manufacturer's instructions (Table 1). Real-time PCR amplification of cDNA was performed using total RNA (15 ng) in a reaction mixture (12 μl) containing TaqMan Universal Master Mix (1 $\times$), primers (900 nmol/L each), and FAM-BHQ1 dual-labeled probe (250 nmol/L). The amplification reactions (with three replicates for each real-time PCR run) were performed with initial hold steps (50°C for 2 minutes, followed by 95°C for 10 minutes) and 40 cycles of a two-step PCR (92°C for 15 s, 60°C for 1 minute). The mRNA level of target gene normalized to an endogenous control (β -actin was used as endogenous reference for hepatocyte growth factor (HGF), fibroblast growth factor (FGF)2, platelet-derived growth factor (PDGF), cyclin-dependent kinase (CDK)1, transforming growth factor (TGF) α , PPAR α , acyl-coenzyme A oxidase 1 (*Acox1*), liver fatty acid binding protein 1 (*Fabp1*), stearoyl-CoA desaturase 1 (*Scd1*), *Cry1*, and *Wee1*, while albumin was used as endogenous reference for brain and muscle Arnt-like protein 1 (*Bmal1*), *Rev-erb α* , *P21*, *Cry2*, *Per1*, *CDK2*, *cyclin A2*, and *cyclin B1*) and relative to a calibrator (the sham-operated mice of the first studied time point in each figure) was calculated by a comparative cycle threshold (C_T) method using a formula: $2^{-\Delta\Delta C_T}$, where $\Delta C_T = C_{T\text{Target}} - C_{T\text{Endogenous control}}$; $\Delta\Delta C_T = \Delta C_{T\text{Experimental group}} - \Delta C_{T\text{Calibrator}}$ (Chemistry Guide, Real-time PCR systems, Applied Biosystems, Foster City, CA). Three to six mice were included at each time point.

Western Blotting

Liver protein (50 to 200 μg) was electrophoresed and transferred to polyvinylidene difluoride membranes.^{7,14} After incubation with primary antibodies (1:1000 dilution) at 4°C , blots were incubated with peroxidase-conjugated secondary antibodies (1:4000 dilution). Immune complexes were visualized by enhanced chemiluminescence

system (Pierce, Rockford, IL). The intensities of the bands were quantified by densitometry with normalization to β -actin protein level using the Quantity One 1-D Analyzer Software (Bio-Rad Laboratories, CA). Antibodies used included: cyclin D1 (DCS6) and Cdk4 (DCS156) from Cell Signaling Technology, MA; Cdk1 P34(17) and cyclin E1 (M-20) from Santa Cruz Biotechnology (CA); and β -actin from Abcam (Cambridge, MA).

Plasmid Constructs

The 10-kb regulatory region (containing two DR-1 elements) from the transcription start site of the *cyclin E1* gene was subcloned into the pGL3 basic luciferase reporter plasmid (Promega Corporation, Madison, WI). PCR was performed with Accu Taq LA DNA Polymerase (Sigma, St. Louis, MO) using sequence-specific primers containing splice sites for *MluI* and *XhoI*. The forward primer was 5'-ATGCACGCGTCACTAGGGCTGGCAGACAAG-3' containing a *MluI* restriction site (bold) and the reverse primer was 5'-GCATCTCGAGCGGAGTTAAGAACCCGTCAT-3' containing an *XhoI* site (bold) for subsequent directional cloning. The PCR product was gel purified, digested with the appropriate restriction enzymes, and ligated into the pGL3-basic plasmid, which was cleaved with the same restriction enzymes. In addition, annealed oligonucleotides containing the putative nuclear receptor binding sites [–AGGTCAAATGTCA–(–9036 to –9048)] and [–AGGTCAAAGGTGG–(–5947 to –5959)] were inserted into *XhoI*-*BglII* digested pGL4.74 [hRluc/TK] vector (Promega Corporation, Madison, WI).

Transient Transfection and Luciferase Activity

Huh7 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal calf serum (Biomed, Foster City, CA) and 100 U/ml penicillin/streptomycin. Approximately 4×10^8 cells per well were plated onto 24-well plates and cultured at 37°C in 5% CO₂ with a relative humidity of 95%. The plated cells were allowed to recover for 1 day before transfection with various reporter vectors and expression plasmids of RXR α alone or RXR α plus PPAR α (provided by Dr. Ronald Evans, Salk Institute, CA) using Lipofectamine 2000 transfection reagent (Promega Corporation, Madison, WI). The tk-(CRBP1)-Luc (provided by Dr. Ronald Evans, Salk Institute, CA) and tk-(PPRE)-Luc (provided by Dr. Frank J. Gonzalez, National Cancer Institute, NIH, MD) vectors served as positive controls for activation of RXR α homodimer and RXR α -PPAR α heterodimer, respectively. For each transfection, herpes simplex virus thymidine kinase promoter-driven *Renilla reniformis* luciferase was used as an internal control for normalization. After transfection, cells were treated with dimethyl sulfoxide (0.1%), 9-*cis*-retinoic acid (9-*cis*-RA), all-trans RA, 13-*cis*-RA, as well as Wy-14,643 (10 μ M/L). Fresh medium containing ligand was provided every 24 hours. Forty-eight hours after treatment, firefly and *Renilla* luciferase activities were measured sequentially using a Dual-Luciferase Reporter assay system (Promega, Madison,

WI) in a single tube TD20/20 luminometer according to the manufacturer's instructions.

Statistical Analysis

Data are presented as means $\pm$ SD ($n = 3$ –6). Statistical analysis was performed using unpaired Student's *t*-tests. Statistical significance was taken at the $P < 0.05$ level.

Results

Impaired Hepatocyte Proliferation in Hepatocyte RXR α -Null Mice after PH

To examine the effect of hepatocyte RXR α on hepatocyte proliferation, Ki-67 immunostaining was performed on liver sections obtained from mice 24 to 120 hours after PH (Figure 1A). Few proliferating cells were noted 24 hours after PH. The number of Ki-67-positive hepatocytes in wild-type mouse livers rapidly increased, and peaked at 36 hours after liver resection (Figure 1B). Proliferation of hepatocytes remained at its peak level until 56 hours and then gradually decreased. In contrast, in regenerating RXR α -null mouse livers, hepatocyte proliferation peaked at 68 hours after PH and sustained at a high level until 72 hours, then decreased. There was proximal 1-day delay in hepatocyte proliferation due to RXR α deficiency; significant higher numbers of proliferating cells were observed in regenerating wild-type than RXR α -null mouse livers from 36 to 56 hours. Consistently, 36 hours after PH, wild-type mice had an increased liver-to-body weight ratio (2.70%) than mutant mice did (2.28%). However, liver-to-body weight ratio was not significantly different at 48 and 56 hours after PH (Figure 1C). At 72 hours post-PH, RXR α -null mouse livers had more Ki-67-positive cells than wild-type mouse livers did, likely leading to the slightly larger liver mass observed in RXR α -null mice at this time point. The liver-to-body weight ratio in wild-type and mutant mice was 3.34% and 3.84%, respectively, at 72 hours. Since liver mass could also be affected by fat accumulation or cell hypertrophy, Ki-67-labeling might be more reliable to monitor cell proliferation and liver growth. These data indicated that hepatocyte proliferation was shifted 24 hours later due to hepatocyte RXR α deficiency.

Impaired Growth Factor Signaling Pathways in Regenerating Hepatocyte RXR α -Null Mouse Livers

HGF is crucial for liver regeneration. After PH, HGF is activated very early and intrahepatic HGF is consumed within the first 3 hours, followed by new HGF synthesis from 3 to 48 hours.¹⁵ In addition, other growth factors including FGF2, PDGF, and TGF α have also been implicated in hepatocyte proliferation during liver regeneration.^{16–19} The impact of RXR α deficiency on the expression of these growth factors was studied during liver regeneration. Our data showed that in contrast to wild-type mouse livers, in which HGF mRNA induction oc-

Figure 1. Defective hepatocyte proliferation in hepatocyte *RXRα*-null mice after PH. **A:** Representative of Ki-67 immunostaining in wild-type and *RXRα*-null mouse livers at indicated time points after PH. **B:** Quantification of Ki-67-positive hepatocytes in at least five microscope fields ($\times 20$ optical field). **C:** Liver-to-body weight ratio (%). Results were expressed as mean $\pm$ SD ($n = 3$ to 6 mice/group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus wild-type mice.

currer 3.5 hours after PH, the induction of HGF mRNA in the mutant mice took place 56 hours after PH, which might account for the delayed proliferation found 72 hours after PH (Figure 2A). Consistently, protein level of HGF was induced in wild-type mouse livers, but not in *RXRα*-null livers, 3.5 hours post PH (Figure 2B). In addition, mRNA levels of HGF were lower in regenerating *RXRα*-null livers than regenerating wild-type mouse livers 0.5 and 3.5 hours after PH (Figure 2A). Induction of the expression of *FGF2* and *PDGF* genes was observed in wild-type mouse livers at 56 hours and 48 hours after PH, respectively, but not in regenerating *RXRα*-null mouse livers. Additionally, when wild-type hepatocytes were actively proliferating (48 and 56 hours after PH), the levels of *FGF2* and *PDGF* mRNA were significantly higher in re-

generating wild-type than mutant mouse livers (Figure 2, C and D). Thus, these changes in gene expression might be in part responsible for the delayed hepatocyte proliferation observed in hepatocyte *RXRα*-null livers.

In contrast to HGF, *FGF2*, and *PDGF*, which had a delayed induction in regenerating mutant mouse livers, the induction of *TGFα* mRNA was consistently found in *RXRα*-null mouse livers 48, 56, 68, and 72 hours after PH; whereas *TGFα* induction was only noted at 56 hours after PH in wild-type mouse livers. Furthermore, significantly higher mRNA levels of *TGFα* were found due to lack of *RXRα* 48 and 72 hours after PH (Figure 2E). These data suggested that *TGFα* might play a more important role in stimulating hepatocyte proliferation in *RXRα*-null mice. Additional study is needed to define the role and the

Figure 2. Aberrant growth factor expression in regenerating RXR α -null (KO) mice after PH. Temporal profile of HGF mRNA level (A), HGF protein level at 3.5 hours (B), and temporal profiles of mRNA levels for FGF2 (C), PDGF (D), and TGF α (E) after sham and PH operation were assessed by real-time PCR and Western blot. Results were expressed as mean $\pm$ SD ($n = 3$ to 6 mice/group). β -actin was used as endogenous reference for HGF, FGF2, PDGF, and TGF α . * $P < 0.05$, ** $P < 0.01$ versus wild-type mice. * $P < 0.05$, ** $P < 0.01$ versus sham-operated wild-type mice. $\$P < 0.05$, $\$\$P < 0.01$, versus sham-operated RXR α -null mice. SM = sham; PH = partial hepatectomy; WT = wild-type; KO = RXR α -null.

underlying mechanisms. These findings indicate multiple signaling pathways orchestrate liver regeneration; when one pathway is compromised, the other pathways will be activated to compensate for the missing signaling. The elevated mRNA levels of HGF at 56 hours, FGF2 and PDGF at 72 hours, and sustained higher levels of TGF α at 48 and 72 hours in RXR α -null livers might account

for the delayed hepatocyte proliferation occurred in RXR α -null mice.

It is important to note that no impaired growth factor signaling was documented in other nuclear receptor knockout mice during liver regeneration indicating the observed phenomenon in the current study might be hepatocyte RXR α specific (Table 2).^{2-4,7}

Impaired Circadian Clock-Controlled Cell Cycle Progression in Regenerating RXR α -Null Livers

Studies have indicated that progression through the cell cycle occurs at specific times of the day/night cycle and hepatic circadian clockwork participates in liver regeneration by controlling the cell-division cycle.^{20,21} In addition, there are direct interactions of RXR α and RAR α with clock and clock-like protein MOP4, which negatively regulates clock/MOP4: Bmal1-mediated clock gene expression in vascular cells.²² To test whether RXR α is required for hepatocyte proliferation via affecting circadian clock-mediated cell cycle division, we examined the mRNA expression profiles of circadian clock genes in both regeneration wild-type and RXR α -null mouse livers.

Deregulation of PPAR α /Bmal1/Rev-erb α /P21 Pathway due to RXR α Deficiency

Bmal1, which is a core clock regulator, is critical for cyclin-dependent kinase inhibitor 1A (P21) expression through the Rora/Rev-erb α pathway.²⁰ P21 negatively regulates G1 phase progression.²⁰ A decreased proliferation was observed in Bmal1-null primary hepatocytes, which have elevated levels of P21.²⁰ PPAR α is a direct regulator of Bmal1 expression in the liver via its direct binding to the Bmal1 promoter.²³ Impaired cell cycle gene regulation was reported in PPAR α -null mouse liver, but whether the delay was due to altered Bmal1-mediated signaling was not established.^{3,4} Since RXR α is an essential partner of PPAR α , we hypothesize that impaired liver regeneration in RXR α -null mice is in part due to deregulation of the PPAR α /Bmal1/Rev-erb α /P21/cell cycle pathway.

Table 2. The Role of Nuclear Receptor in Liver Regeneration

Nuclear receptor knockout mice	Liver regeneration	Growth factor	Circadian clock	Cell cycle	Reference
RXR α (current study)	Delayed	Dependent	Dependent	Dependent	29
RXR α	Liver necrosis occurred due to lack of RXR α . Increased BrdU positive hepatocytes after PH	NR	NR	NR	
FXR	Delayed	Independent	NR	NR	
PXR	Delayed	NR	NR	NR	
PXR	Not delayed	NR	NR	NR	2
PPAR α	Delayed	NR	NR	Cdk2 and Cdk4	3
				P21 and P27	
PPAR α	Delayed	NR	NR	Cyclin D1	4
PPAR α	Not delayed	NR	NR	NR	5, 6
PPAR α	NR	NR	Bmal1	NR	20
CAR	Delayed	NR	NR	NR	2

NR, not reported.

To test this hypothesis, the expression of *PPARα* gene was studied during liver regeneration. At 48 hours after PH, while the wild-type hepatocytes were actively proliferating, the level of *PPARα* mRNA reached its peak. This finding indicates the importance of *PPARα* at this particular time point during regeneration. To monitor the activation of *PPARα*, we studied the expression of *PPARα* target genes *Acox1*, liver *Fabp1*, and *Scd1*.^{24,25} *Fabp1* mRNA level in sham-operated mice was reduced due to *RXRα* deficiency, which was consistent with the basal level change without any treatment reported in our previous publication.¹² In contrast to our previous finding, which showed the basal *PPARα* mRNA levels were increased by twofold due to hepatocyte *RXRα* deficiency, the current study does not observe significant changes in *PPARα* mRNA levels because of *RXRα* deficiency. Since *PPARα* is a circadian gene,²⁶ the variation of *PPARα* mRNA level might be due to the timing when the mice were sacrificed. Forty-eight hours after PH, mRNA levels of *Acox1* (3.2-fold), *Fabp1* (1.9-fold), and *Scd1* (9.2-fold) were up-regulated in wild-type mouse livers, but not in *RXRα*-null livers. As a result, mRNA levels of *Acox1* (2.5-fold), *Fabp1* (2.4-fold), and *Scd1* (2.7-fold) were significantly higher in regenerating wild-type than regenerating mutant mouse livers at this time point (Figure 3A and B). The data suggested that *PPARα* signaling pathway was activated in wild-type mouse livers at this time point. Consistent with this finding, at the same time point, significant induction of *Bmal1* and *Rev-erbα* was observed in wild-type mouse livers, but not in *RXRα*-null livers, after PH. In addition, *Bmal1* and *Rev-erbα* mRNA levels were significantly higher in the regenerating wild-type than the regenerating *RXRα*-null livers. However, at the same time point (48 hours after PH), the level of p21 mRNA peaked in the regenerating *RXRα*-null livers and its level was significantly higher than that of regenerating wild-type mouse livers. Thus, G1 phase progression can be compromised due to elevated P21 (Figure 3, C–E). Take together, a compromise of *PPARα*/*Bmal1*/*Rev-erbα*/P21 pathway in *RXRα*-null mouse livers might contribute to delayed hepatocyte proliferation.

Deregulation of Cry1/Cry2 Expression due to RXRα Deficiency

It has been shown that lack of clock activity (*Cry1*^{−/−}/*Cry2*^{−/−} double mutant animals) leads to decreased cyclin D1 and increased *Wee1* expression levels after PH, and subsequently slows down cell cycle progression.²¹ Thus, *Cry1/2* plays key roles in promoting hepatocyte proliferation. Our data showed the expression patterns of *Cry1/2* mRNA were altered due to lack of hepatocyte *RXRα* (Figure 3F and G). For example, when the wild-type hepatocytes were actively proliferating after PH, the levels of *Cry1* and *Cry2* mRNA were significantly higher in wild-type than in *RXRα*-null mouse livers at 48 hours and 56 hours after PH, respectively. The changes were relatively mild, but the differences reached statistical significance. At 72 hours, *RXRα*-null mouse livers exhibited higher *Cry2* mRNA level, which might help the *RXRα*-null

hepatocytes to start proliferating (Figure 3, F and G). Although the biological meaning of these changes remains to be studied, these data indicate *RXRα* might have an impact on clock gene expression.

Deregulation of Wee1/Per1 Expression due to RXRα Deficiency

Wee1 is a known *Bmal1*/clock target gene, whose product inhibits the G₂/M transition.^{21,27} When *Wee1* reaches its nadir, G₂ phase cells enter mitosis, which occurs at ZT0 (zeitgeber time, ZT0: light on).^{21,27} Thus, in our study, mitosis peaked around 44 hours (6:00 AM in our study) after surgery, which coincided with the low expression of *Wee1* mRNA level observed in regenerating wild-type mouse livers at 48 hours (Figure 3H). In regenerating *RXRα*-null livers, the level of *Wee1* mRNA peaked early (48 hours after PH), which might cause a delay of G₂/M transition and thus reduced cell proliferation at the early studied time points (48 and 56 hours). At 68 hours after PH (6:00 AM, ZT0 of another diurnal cycle), *Wee1* mRNA level reached its nadir in both liver genotypes. However, the hepatic *Wee1* mRNA level was higher in wild-type than in *RXRα* knockout mice, which might explain the increased cell proliferation found at 72 hours after PH in the knockout mice. In addition, *Per1* has also been demonstrated to be involved in the negative regulation of cell cycle targeting at G₂/M transition.²⁸ *Per1* protein has been indicated to induce the checkpoint kinase 2, which results in inhibition of CDK1/Cyclin B1, thus leading to G₂/M suppression.^{20,28} Our data showed that *Per1* mRNA levels were significantly higher in *RXRα*-null mouse livers compared with wild-type mouse livers 48 hours after PH (Figure 3I). Thus, impaired hepatocyte proliferation observed in *RXRα*-null livers at 48 hours after PH might be in part due to deregulated expression of *Wee1* and *Per1*.

Deregulated Cell Cycle Gene Expression in Regenerating Hepatocyte RXRα-Null Mouse Livers

Interference of growth factors and circadian clock gene expression should have an effect on cell cycle progression. We next studied the impact of *RXRα* deficiency on the expression of cell cycle genes in regenerating mouse livers. The expression was studied by real-time PCR and/or Western blot. The focused time points were 48 and 56 hours (at which wild-type and *RXRα*-null livers showed a striking difference in hepatocyte proliferation by Ki-67 staining) as well as 68 and 72 hours after PH (when *RXRα*-null hepatocytes were actively proliferating).

At 48 hours after PH, cyclin D1 level was induced in regenerating wild-type mouse livers and the level was significantly higher than that in *RXRα*-null livers (Figure 4A), consistent with higher *Cry1* mRNA level observed in wild-type mouse livers at the same time point (Figure 3F). In addition, *RXRα* deficiency also resulted in decreased induction of *Cdk4* (Figure 4B). Reduced expression of

Figure 3. Impaired circadian clock-controlled cell cycle progression in regenerating RXR α -null livers. Temporal profile of mRNA level for PPAR α (A) and mRNA levels of PPAR α target genes (Acox1, Fabp1, and Scd1) at 48 hours (B) as well as temporal profiles of mRNA levels for Bmal1 (C), Rev-erb α (D), P21 (E), Cry1 (F), Cry2 (G), Wee1 (H), and Per1 (I) after sham and PH operation were assessed by real-time PCR. Results were expressed as mean $\pm$ SD ($n = 3$ to 6 mice/group). β -actin was used as endogenous reference for PPAR α , Acox1, Fabp1, Scd1, Cry1, and Wee1; while albumin was used as endogenous reference for Bmal1, Rev-erb α , P21, Cry2, and Per1. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, versus wild-type mice. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$, versus sham-operated wild-type mice. $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, and $^{\S\S\S}P < 0.001$, versus sham-operated RXR α -null mice. SM = sham; PH = partial hepatectomy.

cyclin D1 and Cdk4 will definitely hamper the G1 phase progression in RXR α -null livers at 48 hours after PH.

Induction of cyclin E1/Cdk2 was also impaired in regenerating RXR α -null mouse livers. The level of cyclin E1 was significantly induced in regenerating wild-type mouse livers at 48 hours, but not at 72 hours. Surprisingly, at 48 and 72 hours after the surgeries (sham operation and PH), cyclin E1 protein was not detectable in

RXR α -null livers (Figure 4C). These data indicated that hepatocyte RXR α deficiency not only affects the basal cyclin E1 level, but also PH-induced cyclin E1 gene expression. Concurrently, induction of CDK2 mRNA level was observed in regenerating wild-type mouse livers, but not in RXR α -null mouse livers, 48 hours after PH. In addition, Cdk2 mRNA level was much lower in regenerating RXR α -null mouse livers than that in regenerating

Figure 4. Impaired cell cycle progression in hepatocyte RXR α -null (KO) mice after PH. Expressions of cyclin D1 (A), Cdk4 (B), cyclin E1 (C), Cdk2 (D), cyclin A2 (E), cyclin B1 (F), and Cdk1 (G and H) at 48 to 72 hours were assessed by Western blot and/or real-time PCR. Relative protein levels were normalized to β -actin level of the same blot. Representative results are shown. Results were expressed as mean $\pm$ SD ($n = 3$ to 6 mice/group). β -actin was used as endogenous reference for CDK1; while albumin was used as endogenous reference for Cdk2, cyclin A2, and cyclin B1. * $P < 0.05$, ** $P < 0.01$, versus wild-type mice. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, versus sham-operated wild-type mice. \$ $P < 0.05$, \$\$\$ $P < 0.001$, versus sham-operated RXR α -null mice. SM = sham; PH = partial hepatectomy; WT = wild-type; KO = RXR α -null.

wild-type mouse livers at 48 and 56 hours after PH (Figure 4D). Reduced expression and induction of cyclin E1 and Cdk2 will impair G1-S transition in RXR α -null mouse livers.

A strong induction of cyclin A2 mRNA (11.2-fold) was seen 48 hours after PH in wild-type mouse livers (Figure 4E). However, in regenerating RXR α -null mouse livers, only a weak induction of cyclin A2 mRNA level was noted at this time point. Also, significant lower cyclin A2 mRNA level was noted in RXR α -null than wild-type mouse livers 48 hours after PH (Figure 4E), concurrent with reduced Cdk2 mRNA level found in RXR α -null livers at the same time point (Figure 4D), which might lead to impaired progression through the S-phase in RXR α -null livers. At later time points (68 and 72 hours after PH), no significant difference was observed for hepatic Cdk2 and cyclin A2 mRNA levels between wild-type and RXR α -null regenerating livers.

Delayed or impaired induction of cyclin D1/Cdk4 and cyclin E/Cdk2 as well as cyclin A2/Cdk2, together with deregulation of PPAR α /Bmal1/Rev-erb α /P21 pathway, might contribute to delayed progression of cells through G1 and S phase, thus resulting in delayed hepatocyte proliferation in RXR α -null mouse livers.

Cyclin A/Cdk1 and cyclin B1/Cdk1 regulate cell cycle progression during G2/M phase. Significantly higher cyclin B1 and CDK1 mRNA levels were observed in regenerating wild-type than in regenerating RXR α -null mouse livers at 48 hours after PH (Figure 4, F and G), concurrent with elevated Cry1 and reduced Wee1 and Per1 mRNA levels in regenerating wild-type livers at the same time point. CDK1 protein levels were also significantly induced in wild-type mouse livers 48 hours after PH (Figure 4H). However, such induction was not detectable in the mutant mouse livers. Thus, the induction of cyclin B1/Cdk1 after PH might be also hepatocyte RXR α -dependent. Together, reduced expression of cyclin A2/Cdk1 and cyclin B1/Cdk1, as well as elevated expression of Wee1 and Per1, may contribute to delayed hepatocyte proliferation at 48 hours after PH in RXR α -null mice. Interestingly, the mRNA levels of cyclin B1 were higher in regenerating RXR α -null than wild-type livers at 68 hours, which was accompanied with increased protein level of Cdk1 at 72 hours. The higher expression levels of cyclin B1 and Cdk1 in regenerating mutant mice might allow hepatocytes to start proliferation at later time points.

These results clearly indicate that the expression of many cell cycle-related genes and proteins is deregulated

lated, which in turn affects cell cycle progression, in regenerating hepatocyte RXR α -null mouse livers.

Positive Regulation of Cyclin E1 Transcription by Retinoic Acid Signaling

Our data showed that the level of cyclin E1 was reduced due to hepatocyte RXR α deficiency. In addition, PH-induced cyclin E1 expression was not found during hepatocyte proliferation (48 hours after PH) due to hepatocyte RXR α deficiency (Figure 4C). Thus, we hypothesize that *cyclin E1* gene transcription might be regulated by RXR α , which might contribute to liver regeneration. Transient transfection assays were performed to determine whether activation of RXR α pathway by retinoic acid could regulate the transcription of the *cyclin E1* gene.

As shown in Figure 5A, when RXR α alone was overexpressed, 9-*cis*-RA and all-trans RA, but not 13-*cis*-RA, induced the Cyclin E1 promoter driven luciferase activity by 4.2- and 2.1-fold, respectively. In addition, induced reporter activity was observed for -9036DR1-luc construct, but not -5947DR1-luc construct, after 9-*cis*-RA or all-trans RA treatment. In contrast, 9-*cis*-RA-mediated induction of luciferase activity was not found when PPAR α plus RXR α was co-expressed (Figure 5B). Activation of PPAR α /RXR α by Wy-14,643 did not result in induced luciferase activity either. These findings suggest that via RXR α homodimer, but not PPAR α /RXR α heterodimer, all-trans RA or 9-*cis*-RA can directly regulate the transcription of the *cyclin E1* gene. In addition, the DR1 motif located at -9036 upstream from the transcription start site of the *cyclin E1* gene is responsible for mediating the effect of RA in induction of the *cyclin E1* gene. For positive controls, significant induction of luciferase activity for CRBP-II-luc and PPRE-luc reporter constructs was observed after 9-*cis*-RA and Wy-14,643 treatment, respectively.

Discussion

The present study demonstrates that hepatocyte RXR α is required for signaling pathways involved in liver regeneration. The following summarizes our findings: (1) RXR α deficiency results in a delay in hepatocyte proliferation; (2) growth factor-mediated signaling pathways are altered because of hepatocyte RXR α deficiency, which in part contributes to delayed cell proliferation; (3) lack of RXR α also resulted in impaired cell cycle progression due to suppressed activation of cell cycle regulators and deregulation of circadian clock-mediated cell division; and (4) via RXR α homodimer, but not RXR α /PPAR α heterodimer, all-trans RA and 9-*cis*-RA can positively regulate the transcription of the *cyclin E1* gene. Thus, lack of RXR α delayed normal progression of hepatocyte proliferation in response to liver mass loss. At later stages (72 hours after PH), RXR α signaling is compensated by other mechanisms and hepatocyte proliferation is observed in hepatocyte RXR α -null mice, which supports the notion

Figure 5. Identification of RXR α homodimer as a positive and direct regulator of *Cyclin E1* transcription. Huh7 cells were transiently transfected with the reporter construct and expression plasmids (**A**, RXR α homodimer; **B**, RXR α -PPAR α heterodimer) as shown. For each transfection, herpes simplex virus thymidine kinase promoter-driven *Renilla reniformis* luciferase was used as an internal control for normalization. Twenty-four hours after transfection, cells were subjected to respective ligand treatment at 10 μ M/L for 48 hours. Then cells were harvested and firefly and renilla luciferase activities were determined using a single tube TD20/20 luminometer. Results were expressed as mean $\pm$ SD ($n = 3$). * $P < 0.05$ and ** $P < 0.01$ versus dimethyl sulfoxide.

that liver regeneration is orchestrated by multiple signaling pathways.¹

The current study expands the roles of nuclear receptors in liver regeneration. Several studies have documented the crucial roles of nuclear receptors that heterodimerize with RXR α in liver regeneration^{2-4,7,29} (Table 2). Constitutive androstane receptor has been indicated to regulate early phase of hepatic regeneration in response to PH. Absence of constitutive androstane receptor resulted in a modest decrease in liver growth at day 1

combined with delayed DNA replication.² FXR, a mediator of bile acid homeostasis, has been shown to be necessary for normal liver regeneration. FXR deficiency results in significant reduction in liver growth, which is accompanied by decreased DNA synthesis 3 days after PH. However, at later stages, FXR-null mice had a rapid liver growth and no difference was found between wild-type and FXR-null mouse livers 7 days after PH.² The role of PXR in regulating PH-induced liver regeneration has also been investigated. Using a percentage of original liver weight as a liver growth index, no defect in liver growth was detected in PXR-null mice at the early phase of liver regeneration.² However, delayed liver regeneration was observed in PXR-null mice in our previous study associated with Stat3 inactivation, in which more studied time points were included and liver-to-body weight ratio was used to measure liver growth.⁷ The same genetic background of PXR-null mice was used in both studies. PPAR α is necessary for activation and membrane localization of Ras after PH, which is involved in the activation of Cdk5 and initiation of G1/S phase transition.³ PPAR α -null mice exhibited impaired liver regeneration within 48 hours after PH, but regeneration caught up 72 hours post-PH.^{3,4}

The current paper demonstrates that hepatocyte RXR α is essential for multiple signaling pathways that dictate normal progression of liver regeneration. The observed impairment of growth factor induction in our study might be specifically due to hepatocyte RXR α deficiency, since such effects were not observed in mice lacking other nuclear receptors. However, since *Bmal1* is a direct PPAR α target gene,²⁰ hepatocyte RXR α could regulate circadian clock gene expression through coupling with PPAR α or other signaling pathways and subsequently have an impact on cell cycle progression by targeting p21. In regenerating PPAR α knockout mouse livers, the expression of cyclin D1/Cdk4, Cdk2, and p27 was deregulated.^{3,4} Thus, *cyclin D1/Cdk4* and *Cdk2* gene expressions were commonly deregulated in regenerating hepatocyte PPAR α - and RXR α -deficient mouse livers. Based on these findings, it is possible that deregulation of G1-S transition regulated by cyclin D1/Cdk4 and Cdk2 in RXR α -null mouse livers during liver regeneration is PPAR α /RXR α dependent. Whether the deregulation of cyclin A2 and cyclin B1/Cdk1 is RXR α specific or PPAR α /RXR α dependent requires more investigation. In addition, retinoic acid can positively regulate *cyclin E1* transcription via activation of RXR α homodimer. As such, hepatocyte RXR α has an impact on G1, G2, and M checkpoints and can be considered as a cell cycle modulator during liver regeneration. The identification of the roles of these nuclear receptors in liver regeneration provides considerable insights into the molecular mechanisms by which the liver is able to restore its original mass and function in response to liver mass loss.

Additionally, our data showed that the levels of hepatic PPAR α mRNA after PH fluctuated depending on when the mice were sacrificed with the levels elevated during the daytime but decreased at night. The pattern of expression is similar to that observed in normal liver.³⁰ Profiling the time course of circadian clock gene expres-

sion in both genotypes of mouse livers revealed that the rhythmic gene expression pattern was not RXR α -dependent. RXR α -null mice displayed an expression pattern that was basically the same as their wild-type littermates for most of the hepatic clock genes except *Cry2*. This is reasonable since subsidiary circadian oscillators in many peripheral cell types including hepatocytes are synchronized by the master pacemaker in the suprachiasmatic nucleus of the brain in mammals.³¹ However, average expression levels and fluctuation of those clock-controlled genes after PH were changed due to the lack of RXR α , indicating that RXR α might be involved in the mediation of circadian rhythm to some extent. The underlying molecular mechanisms need to be further investigated.

The involvement of RXR α in liver regeneration has been examined by Imai's study,²⁹ in which diminished hepatocyte lifespan and impaired hepatocyte regenerative capacity were reported. In the animal models used by Imai et al,²⁹ RXR α gene mutation leads to liver injury, increased hepatocyte proliferation, and impaired liver regeneration. Thus, increased hepatocyte proliferation during liver regeneration is likely due to a compensatory growth because of liver injury. However, the hepatocyte RXR α -deficient mouse model established by us does not have liver injury, which was monitored by ALT level and liver histology^{14,32}; no liver injury was observed even when the mice were 24 months old (our unpublished finding). Our published data showed that hepatocyte RXR α deficiency results in changes in basal mRNA levels of many genes that are directly regulated by RXR α and its heterodimeric partners,^{12,33} which confirmed the validity of the model established by us. Thus, the delayed liver regeneration observed in RXR α -null mice in our study is not relevant to liver injury. Furthermore, hepatocyte RXR α deficiency does not seem to have an effect on cell proliferation since the number of Ki-67-positive cells in the liver was not different between sham-operated wild-type and sham-operated hepatocyte RXR α -null mice (data not shown). The reason for the discrepancy between our and Imai's findings is not clear. It could be due to a different region of the RXR α gene was deleted or a different genetic background of the mice was used in generating the RXR α -null mice. Retinoic acids (9-*cis*-RA and all-*trans* RA) are known mitogens in rodent liver.^{34,35} Our data showed delayed liver regeneration due to hepatocyte RXR α deficiency is consistent with the mitogenic effect of RAs *in vivo*.

One limitation of the present study is that some conclusions are based on mRNA analysis and the findings should be validated at the protein level. However, our overall finding indicated that hepatocyte proliferation was shifted 24 hours later due to hepatocyte deficiency. Deregulation of hepatocyte growth factor signaling pathway, impaired circadian clock-mediated cell cycle progression as well as irregular cell cycle gene expression in part account for such delay. Thus, hepatocyte RXR α is required for multiple signaling pathways involved in hepatocyte proliferation during liver regeneration.

Acknowledgments

We thank Dr. Ivan Damjanov for his critics and Ms. Barbara Brede for editing the manuscript.

References

1. Michalopoulos GK: Liver regeneration. *J Cell Physiol* 2007, 213:286–300
2. Huang W, Ma K, Zhang J, Qatanani M, Cuvillier J, Liu J, Dong B, Huang X, Moore DD: Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. *Science* 2006, 312:233–236
3. Wheeler MD, Smutney OM, Check JF, Rusyn I, Schulte-Hermann R, Thurman RG: Impaired Ras membrane association and activation in PPARalpha knockout mice after partial hepatectomy. *Am J Physiol Gastrointest Liver Physiol* 2003, 284:G302–G312
4. Anderson SP, Yoon L, Richard EB, Dunn CS, Cattley RC, Corton JC: Delayed liver regeneration in peroxisome proliferator-activated receptor-alpha-null mice. *Hepatology* 2002, 36:544–554
5. Newberry EP, Kennedy SM, Xie Y, Luo J, Stanley SE, Semenkovich CF, Crooke RM, Graham MJ, Davidson NO: Altered hepatic triglyceride content after partial hepatectomy without impaired liver regeneration in multiple murine genetic models. *Hepatology* 2008, 48:1097–1105
6. Rao MS, Peters JM, Gonzalez FJ, Reddy JK: Hepatic regeneration in peroxisome proliferator-activated receptor alpha-null mice after partial hepatectomy. *Hepatol Res* 2002, 22:52–57
7. Dai G, He L, Bu P, Wan YJ: Pregnenal X receptor is essential for normal progression of liver regeneration. *Hepatology* 2008, 47:1277–1287
8. Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong ES, Oro AE, Kakizuka A, Evans RM: Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev* 1992, 6:329–344
9. Germain P, Chambon P, Eichele G, Evans RM, Lazar MA, Leid M, De Lera AR, Lotan R, Mangelsdorf DJ, Gronemeyer H: International Union of Pharmacology LXIII. Retinoid X receptors. *Pharmacol Rev* 2006, 58:760–772
10. Kastner P, Grondona JM, Mark M, Gansmuller A, LeMeur M, Decimo D, Vonesch JL, Dolle P, Chambon P: Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* 1994, 78:987–1003
11. Sucov HM, Dyson E, Gumeringer CL, Price J, Chien KR, Evans RM: RXR alpha mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes Dev* 1994, 8:1007–1018
12. Wan YJ, An D, Cai Y, Repa JJ, Hung-Po Chen T, Flores M, Postic C, Magnuson MA, Chen J, Chien KR, French S, Mangelsdorf DJ, Sucov HM: Hepatocyte-specific mutation establishes retinoid X receptor alpha as a heterodimeric integrator of multiple physiological processes in the liver. *Mol Cell Biol* 2000, 20:4436–4444
13. Greene AK, Puder M: Partial hepatectomy in the mouse: technique and perioperative management. *J Invest Surg* 2003, 16:99–102
14. Gyamfi MA, He L, French SW, Damjanov I, Wan YJ: Hepatocyte retinoid X receptor alpha-dependent regulation of lipid homeostasis and inflammatory cytokine expression contributes to alcohol-induced liver injury. *J Pharmacol Exp Ther* 2008, 324:443–453
15. Padiaditakis P, Lopez-Talavera JC, Petersen B, Monga SP, Michalopoulos GK: The processing and utilization of hepatocyte growth factor/scatter factor following partial hepatectomy in the rat. *Hepatology* 2001, 34:688–693
16. Michalopoulos GK: HGF in liver regeneration and tumor promotion. *Prog Clin Biol Res* 1995, 391:179–185
17. Tomiya T, Ogata I, Fujiwara K: Transforming growth factor alpha levels in liver and blood correlate better than hepatocyte growth factor with hepatocyte proliferation during liver regeneration. *Am J Pathol* 1998, 153:955–961
18. Sturm J, Keese M, Zhang H, Bonninghoff R, Magdeburg R, Vajkoczy P, Dono R, Zeller R, Gretz N: Liver regeneration in FGF-2-deficient mice: vEGF acts as potential functional substitute for FGF-2. *Liver Int* 2004, 24:161–168
19. Murata S, Ohkohchi N, Matsuo R, Ikeda O, Myronovych A, Hoshi R: Platelets promote liver regeneration in early period after hepatectomy in mice. *World J Surg* 2007, 31:808–816
20. Grechez-Cassiau A, Rayet B, Guillaumond F, Teboul M, Delaunay F: The circadian clock component BMAL1 is a critical regulator of p21WAF1/CIP1 expression and hepatocyte proliferation. *J Biol Chem* 2008, 283:4535–4542
21. Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F, Okamura H: Control mechanism of the circadian clock for timing of cell division in vivo. *Science* 2003, 302:255–259
22. McNamara P, Seo SB, Rudic RD, Sehgal A, Chakravarti D, FitzGerald GA: Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. *Cell* 2001, 105:877–889
23. Canaple L, Rambaud J, Dkhis-Benyahya O, Rayet B, Tan NS, Michalik L, Delaunay F, Wahli W, Laudet V: Reciprocal regulation of brain and muscle Arnt-like protein 1 and peroxisome proliferator-activated receptor alpha defines a novel positive feedback loop in the rodent liver circadian clock. *Mol Endocrinol* 2006, 20:1715–1727
24. Desvergne B, Wahli W: Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* 1999, 20:649–688
25. Miller CW, Ntambi JM: Peroxisome proliferators induce mouse liver stearoyl-CoA desaturase 1 gene expression. *Proc Natl Acad Sci USA* 1996, 93:9443–9448
26. Yang X, Downes M, Yu RT, Bookout AL, He W, Straume M, Mangelsdorf DJ, Evans RM: Nuclear receptor expression links the circadian clock to metabolism. *Cell* 2006, 126:801–810
27. Schibler U: Circadian rhythms. Liver regeneration clocks on. *Science* 2003, 302:234–235
28. Gery S, Komatsu N, Baldjyan L, Yu A, Koo D, Koeffler HP: The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells. *Mol Cell* 2006, 22:375–382
29. Imai T, Jiang M, Kastner P, Chambon P, Metzger D: Selective ablation of retinoid X receptor alpha in hepatocytes impairs their lifespan and regenerative capacity. *Proc Natl Acad Sci USA* 2001, 98:4581–4586
30. Yang X, Zhang YK, Esterly N, Klaassen CD, Wan YJ: Gender disparity of hepatic lipid homeostasis regulated by the circadian clock. *J Biochem* 2009, 145:609–623
31. Schibler U, Sassone-Corsi P: A web of circadian pacemakers. *Cell* 2002, 111:919–922
32. Gyamfi MA, Wan YJ: Mechanisms of resistance of hepatocyte retinoid X receptor a-null mice to WY-14,643-induced hepatocyte proliferation and cholestasis. *J Biol Chem* 2009, 284:9321–9330
33. Wan YJ, Cai Y, Lungu W, Fu P, Locker J, French S, Sucov HM: Peroxisome proliferator-activated receptor alpha-mediated pathways are altered in hepatocyte-specific retinoid X receptor alpha-deficient mice. *J Biol Chem* 2000, 275:28285–28290
34. Ohmura T, Columbano GL, Columbano A, Katyal SL, Locker J, Shinozuka H: 9-cis retinoic acid is a direct hepatocyte mitogen in rats. *Life Sci* 1996, 58:PL211–P216
35. Ledda-Columbano GM, Pibiri M, Molotzu F, Cossu C, Sanna L, Simbula G, Perra A, Columbano A: Induction of hepatocyte proliferation by retinoic acid. *Carcinogenesis* 2004, 25:2061–2066