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

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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 (RXRα) is an essential partner of these nuclear receptors. Therefore, we studied the role of hepatocyte RXRα in liver regeneration using partial hepatectomy model. The results showed that hepatocyte RXRα 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 RXRα 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 RXRα. Furthermore, the peroxisome proliferator-activated receptor α (Pparg)-mediated Ras and Rhoa membrane association plays a key role in early DNA synthesis and cell proliferation. Disruption of PPARα-mediated lipid signaling pathway transiently impairs the initiation of liver regeneration. However, other studies show that PPARα deficiency does not affect liver regeneration following PH. This discrepancy might be in part due to differences in the genetic backgrounds of the mice used. Pregnan 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. Delayed hepatocyte proliferation is also noted in constitutive androstane receptor (also known as Nr1i3) knockout mice.

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. 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. Peroxisome proliferator-activated receptor α (PPARα)-mediated lipid signaling pathway transiently impairs the initiation of liver regeneration. However, other studies show that PPARα deficiency does not affect liver regeneration following PH. This discrepancy might be in part due to differences in the genetic backgrounds of the mice used. Pregnan 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. Delayed hepatocyte proliferation is also noted in constitutive androstane receptor (also known as Nr1i3) knockout mice.

Retinoid X receptor α (RXRα) is the most abundant RXR among the three RXR isoforms (α, β, and γ). RXRα forms dimers with FXR, PPARα, constitutive androstane receptor, and PXR and regulates gene transcription. Supported by National Institutes of Health grants CA53596 and COBRE P20 RR021940. Accepted for publication September 29, 2009.

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Lack of RXRα impairs activation of its partners and subsequently affects the downstream cascades. In addition, RXRα forms homodimers and heterodimers with retinoic acid receptors. RXRα is essential in the transduction of a retinoid signal required for myocardial development and ocular morphogenesis, suggesting that RXRα is critical for cell proliferation and differentiation. Lack of RXRα results in a transient delay in liver growth during embryonic development. In the current study, we investigated the modulation of liver regeneration by RXRα 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 RXRα-deficient mice (3 to 4 months old) were used for partial hepatectomy. Hepatocyte RXRα-deficient mice were generated by deletion of exon 4 of the RXRα gene using albumin-cre/loxP-mediated recombination. The control mice had RXRα 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. 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 (×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×), 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), fibrolast growth factor (FGF2), platelet-derived growth factor (PDGF), cyclin-dependent kinase (CDK1), transforming growth factor (TGF)α, PPARα, acyl-coenzyme A oxidase 1 (Acox1), liver fatty acid binding protein 1 (Fabp1), stearoyl-CoA desaturase 1 (Scd1), Cry1, and P21) was determined using a comparative cycle threshold (CT) method using a formula: 2−ΔΔCT, where ΔΔCT = CTExperimental − CTEndogenous control − ΔCT Experimental group − ΔCT 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. 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 \( \beta \)-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 \( \beta \)-actin from Abcam (Cambridge, MA).

**Plasmid Constructs**

The 10-kb regulatory region (containing two DR-1 elements) from the transcription start site of the *cyclophilin 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 Xhol. The forward primer was 5’-ATGCACCGGTCACATGGGCTGAGAAG-3’ containing a *MluI* restriction site (bold) and the reverse primer was 5’-GCATCTGGAGCCGAGTTAAGAACCCGTCAT-3’ containing an Xhol 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 [\( \approx \)AGGTCAATTGCTA-(−9036 to −9048)] and [\( \approx \)AGGTCAAAAGTGQ-(−5947 to −5959)] were inserted into Xhol-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 × 10^4 cells per well were plated onto 24-well plates and cultured at 37°C in 5% CO\(_2\) 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 RXRa alone or RXRa plus PPAR\( \alpha \) (provided by Dr. Ronald Evans, Salk Institute, CA) using Lipofectamine 2000 transfection reagent (Promega Corporation, Madison, WI). The tk-(CRBPII)-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 RXRa homodimer and RXRa-PPAR\( \alpha \) 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 μmol/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 ± 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 RXRa-Null Mice after PH**

To examine the effect of hepatocyte RXRa 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 RXRa-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 RXRa deficiency; significantly higher numbers of proliferating cells were observed in regenerating wild-type than RXRa-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, RXRa-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 RXRa-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 RXRa deficiency.

**Impaired Growth Factor Signaling Pathways in Regenerating Hepatocyte RXRa-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\( \alpha \) have also been implicated in hepatocyte proliferation during liver regeneration.\(^{16–19}\) The impact of RXRa 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-
curred 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 regenerating 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
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 at 48 and 72 hours in RXR-null livers might account for the delayed hepatocyte proliferation occurred in RXR-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-specific (Table 2).

Impaired Circadian Clock-Controlled Cell Cycle Progression in Regenerating RXR-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. In addition, there are direct interactions of RXR and RAR with clock and clock-like protein MOP4, which negatively regulates clock/MOP4:Bmal1-mediated clock gene expression in vascular cells. To test whether RXR 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-null mouse livers.

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

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

Table 2. The Role of Nuclear Receptor in Liver Regeneration

<table>
<thead>
<tr>
<th>Nuclear receptor knockout mice</th>
<th>Liver regeneration</th>
<th>Growth factor</th>
<th>Circadian clock</th>
<th>Cell cycle</th>
<th>Reference</th>
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<tr>
<td>RXRα (current study)</td>
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<td>NR</td>
<td>Dependent</td>
<td>Dependent</td>
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<tr>
<td>RXRα</td>
<td>Liver necrosis occurred due to lack of RXRα, increased BrdU positive hepatocytes after PH</td>
<td>NR</td>
<td>Dependent</td>
<td>Dependent</td>
<td>29</td>
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<tr>
<td>FXR</td>
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<td>Independent</td>
<td>NR</td>
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<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>7</td>
</tr>
<tr>
<td>PXR</td>
<td>Not delayed</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>2</td>
</tr>
<tr>
<td>PPARα</td>
<td>Delayed</td>
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<td>NR</td>
<td>Cdk2 and Cdk4</td>
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<tr>
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<td>NR</td>
<td>NR</td>
<td>P21 and P27</td>
<td>4</td>
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<tr>
<td>PPARα</td>
<td>Not delayed</td>
<td>NR</td>
<td>NR</td>
<td>Cyclin D1</td>
<td>5, 6</td>
</tr>
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<td>NR</td>
<td>NR</td>
<td>Bmal1</td>
<td>20</td>
</tr>
<tr>
<td>CAR</td>
<td>Delayed</td>
<td>NR</td>
<td>NR</td>
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<td>2</td>
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</table>

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-erba was observed in wild-type mouse livers, but not in RXRα-null livers, after PH. In addition, Bmal1 and Rev-erba 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-erba/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
cyclin D1 and Cdk4 will definitely hamper the G1 phase progression in RXRα-null livers at 48 hours after PH. Induction of cyclin E1/Cdk2 was also impaired in regenerating RXRα-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 RXRα-null livers (Figure 4C). These data indicated that hepatocyte RXRα 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 RXRα-null mouse livers, 48 hours after PH. In addition, Cdk2 mRNA level was much lower in regenerating RXRα-null mouse livers than that in regenerating wild-type mouse livers at 48 hours after PH. These findings suggest that RXRα deficiency impairs the induction of CDK2 mRNA level in regenerating livers.

Figure 3. Impaired circadian clock-controlled cell cycle progression in regenerating RXRα-null livers. Temporal profile of mRNA level for PPARα (A) and mRNA levels of PPARα target genes (Acox1, Fabp1, and Scd1) at 48 hours (B) as well as temporal profiles of mRNA levels for Bmal1 (C), Rev-erbα (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 ± SD (n = 3 to 6 mice/group). β-actin was used as endogenous reference for PPARα, Acox1, Fabp1, Scd1, Cry1, and Wee1; while albumin was used as endogenous reference for Bmal1, Rev-erbα, 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. $P < 0.05, §§P < 0.01, and §§§P < 0.001, versus sham-operated RXRα-null mice. SM = sham, PH = partial hepatectomy.
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 RXRα-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 RXRα-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 RXRα-null than wild-type mouse livers 48 hours after PH (Figure 4E), concurrent with reduced Cdk2 mRNA level found in RXRα-null livers at the same time point (Figure 4D), which might lead to impaired progression through the S-phase in RXRα-null mice. 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 RXRα-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 PPARα/Bmal1/Rev-erba/P21 pathway, might contribute to delayed progression of cells through G1 and S phase, thus resulting in delayed hepatocyte proliferation in RXRα-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 RXRα-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 mice 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 RXRα-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 RXRα-null mice. Interestingly, the mRNA levels of cyclin B1 were higher in regenerating RXRα-null mice 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 deregu-
lated, which in turn affects cell cycle progression, in regenerating hepatocyte RXRα-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 RXRα deficiency. In addition, PH-induced cyclin E1 expression was not found during hepatocyte proliferation (48 hours after PH) due to hepatocyte RXRα deficiency (Figure 4C). Thus, we hypothesize that cyclin E1 gene transcription might be regulated by RXRα, which might contribute to liver regeneration. Transient transfection assays were performed to determine whether activation of RXRα pathway by retinoic acid could regulate the transcription of the cyclin E1 gene.

As shown in Figure 5A, when RXRα 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 treatment. In contrast, 9-cis-RA-mediated induction of luciferase activity was not found when PPARα plus RXRα was co-expressed (Figure 5B). Activation of PPARα/RXRα by Wy-14,643 did not result in induced luciferase activity either. These findings suggest that via RXRα homodimer, but not PPARα/RXRα 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 CRBPII-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 RXRα is required for signaling pathways involved in liver regeneration. The following summarizes our findings: (1) RXRα deficiency results in a delay in hepatocyte proliferation; (2) growth factor-mediated signaling pathways are altered because of hepatocyte RXRα deficiency, which in part contributes to delayed cell proliferation; (3) lack of RXRα 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 RXRα homodimer, but not RXRα/PPARα heterodimer, all-trans RA and 9-cis-RA can positively regulate the transcription of the cyclin E1 gene. Thus, lack of RXRα delayed normal progression of hepatocyte proliferation in response to liver mass loss. At later stages (72 hours after PH), RXRα signaling is compensated by other mechanisms and hepatocyte proliferation is observed in hepatocyte RXRα-null mice, which supports the notion 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 RXRα in liver regeneration2-4,7,29 (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. 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. 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. 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. The same genetic background of PXR-null mice was used in both studies. PPARα is necessary for activation and membrane localization of Ras after PH, which is involved in the activation of Cdns and initiation of G1/S phase transition. PPARα-null mice exhibited impaired liver regeneration within 48 hours after PH, but regeneration caught up 72 hours post-PH.

The current paper demonstrates that hepatocyte RXRα 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 RXRα deficiency, since such effects were not observed in mice lacking other nuclear receptors. However, since Brn1 is a direct RXRα target gene, hepatocyte RXRα could regulate circadian clock gene expression through coupling with PPARα or other signaling pathways and subsequently have an impact on cell cycle progression by targeting p21. In regenerating PPARα knockout mouse livers, the expression of cyclin D1/Cdk4, Cdk2, and p27 was deregulated. Thus, cyclin D1/Cdk4 and Cdk2 gene expressions were commonly deregulated in regenerating hepatocyte PPARα- and RXRα-deficient mouse livers. Based on these findings, it is possible that deregulation of G1/S transition regulated by cyclin D1/Cdk4 and Cdk2 in RXRα-null mouse livers during liver regeneration is PPARα/RXRα dependent. Whether the deregulation of cyclin A2 and cyclin B1/Cdk1 is RXRα specific or PPARα/RXRα dependent requires more investigation. Additionally, retinoic acid can positively regulate cyclin E1 transcription via activation of RXRα homodimer. As such, hepatocyte RXRα 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 PPARα 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. Profiling the time course of circadian clock gene expression in both genotypes of mouse livers revealed that the rhythmic gene expression pattern was not RXRα-dependent. RXRα-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. However, average expression levels and fluctuation of those clock-controlled genes after PH were changed due to the lack of RXRα, indicating that RXRα might be involved in the mediation of circadian rhythm to some extent. The underlying molecular mechanisms need to be further investigated.

The involvement of RXRα in liver regeneration has been examined by Imai’s study, in which diminished hepatocyte lifespan and impaired hepatocyte regenerative capacity were reported. In the animal models used by Imai et al., RXRα 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 RXRα-deficient mouse model established by us does not have liver injury, which was monitored by ALT level and liver histology; no liver injury was observed even when the mice were 24 months old (our unpublished finding). Our published data showed that hepatocyte RXRα deficiency results in changes in basal mRNA levels of many genes that are directly regulated by RXRα and its heterodimeric partners, which confirmed the validity of the model established by us. Thus, the delayed liver regeneration observed in RXRα-null mice in our study is not relevant to liver injury. Furthermore, hepatocyte RXRα 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 RXRα-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 RXRα gene was deleted or a different genetic background of the mice was used in generating the RXRα-null mice. Retinoic acids (9-cis-RA and all-trans RA) are known mitogens in rodent liver. Our data showed delayed liver regeneration due to hepatocyte RXRα 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. De-regulation 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 RXRα is required for multiple signaling pathways involved in hepatocyte proliferation during liver regeneration.
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References