BASIC STUDIES

Retinoic acid signalling induces the differentiation of mouse fetal liver-derived hepatic progenitor cells

Jiayi Huang1,2*, Yang Bi1,2*, Gao-Hui Zhu1,2, Yun He1,2, Yuxi Su1,2, Bai-Cheng He1,2, Yi Wang1,2, Quan Kang1,2, Liang Chen1,2, Guo-Wei Zuo1,2, Qiong Luo1,2, Qiong Shi1,2, Bing-Qiang Zhang1,2, Ailong Huang1, Lan Zhou1,2, Tao Feng1,2, Hue H. Luu2, Rex C. Haydon2, Tong-Chuan He1,2 and Ni Tang1,2

1 Key Laboratory of Diagnostic Medicine designated by the Ministry of Education of China, The Affiliated Hospitals of Chongqing Medical University, Chongqing, China
2 Molecular Oncology Laboratory, Department of Surgery, The University of Chicago Medical Center, Chicago, IL, USA

Abstract

Background: Hepatic progenitor cells (HPCs) can be isolated from fetal liver and extrahepatic tissues. Retinoic acid (RA) signalling plays an important role in development, although the role of RA signalling in liver-specific progenitors is poorly understood. Aims: We sought to determine the role of RA in regulating hepatic differentiation. Methods: RNA was isolated from liver tissues of various developmental stages. Liver marker expression was assessed by reverse transcriptase-polymerase chain reaction and immunofluorescence staining. Reversibly immortalized HPCs derived from mouse embryonic day 14.5 (E14.5) liver (aka, HP14.5) were established. Albumin promoter-driven reporter (Alb-GLuc) was used to monitor hepatic differentiation. Glycogen synthesis was assayed as a marker for terminal hepatic differentiation. Results: Retinoic acid receptor (RAR)-α, retinoid X receptor (RXR)-α and RXR-γ expressed in E12.5 to postnatal day 28 liver samples. Expression of RAR-β and RXR-β was low perinatally, whereas RAR-γ was undetectable in prenatal tissues and increased postnatally. Retinal dehydrogenase 1 and 2 (Raldh1 and Raldh2) were expressed in all tissues, while Raldh3 was weakly expressed in prenatal samples but was readily detected postnatally. Nuclear receptor corepressors were highly expressed in all tissues, while expression of nuclear co-activators decreased in perinatal tissues and increased after birth. HP14.5 cells expressed high levels of early liver stem cell markers. Expression of RA signalling components and coregulators was readily detected in HP14.5. RA was shown to induce Alb-GLuc activity and late hepatocyte markers. RA was further shown to induce glycogen synthesis in HP14.5 cells, an important function of mature hepatocytes. Conclusions: Our results strongly suggest that RA signalling may play an important role in regulating hepatic differentiation.

Hepatocytes differentiate from the endoderm during embryonic development (1–3). Liver derivation from the foregut endoderm occurs around somite stages 5–6 as a result of signalling from mesoderm in the form of fibroblast growth factors and BMP4 (2, 3). Liver has a regenerative capacity following partial hepatectomy or injury (3–7). The majority of liver-derived stem cell research has focussed on either fetal-derived hepatic stem cells or oval cells (2, 8–12). During embryogenesis, the cellular constituent of the liver is composed of hepatoblasts that are defined as the bipotential precursors for hepatocytes and cholangiocytes (3, 8, 13). Hepatoblasts begin to differentiate at embryonic day 14 (E14) into hepatocytes and bile duct cells (cholangiocytes) (3, 12). Hepatic progenitor cells (HPCs) can be identified and isolated from extrahepatic adult tissues (5, 9, 14–16).

Retinoic acid (RA) plays a critical role in embryonic development and function maintenance of vital organs in adult (17, 18). RA is formed solely from retinaldehyde (Rald), which is derived from vitamin A. The metabolism of vitamin A and the diverse effects of its metabolites are tightly controlled by distinct retinoid-generating enzymes, retinoid-binding proteins and retinoid-activated nuclear receptors (17, 19). RA regulates differentiation and metabolism by serving as a ligand for two families of nuclear receptors, the retinoic acid receptors (RAR-α, RAR-β and RAR-γ) that bind the abundant form of RA...
known as all-trans-retinoic acid (ATRA) and the retinoid X receptors (RXR-\(\alpha\), RXR-\(\beta\) and RXR-\(\gamma\)) that bind an isomer known as 9-cis-retinoic acid (9CRA) (20, 21), normally undetectable except when vitamin A is present in excess (22). RXR forms heterodimers with RAR and several other nuclear receptors when bound to DNA, suggesting that RXR may function as a scaffold protein to facilitate DNA binding for several types of nuclear receptors (20, 21). In vivo studies have demonstrated that ligand binding to just the RAR portion of RAR/RXR heterodimers is sufficient and necessary to rescue a lethal defect in RA synthesis, whereas ligand binding to RXR does not rescue the defect and is unnecessary (22). RA binding to RAR/RXR heterodimers bound to a regulatory DNA element leads to a cascade of events resulting in recruitment of transcriptional co-activators and initiation of transcription (20, 23). As for other members of the nuclear receptor superfamily, RA-induced transcriptional activity is tightly regulated by nuclear corepressors (NCORs) and nuclear receptor co-activators (NCOAs) (24). Genetic manipulations in animals have revealed that RA signalling is important for the development of the forebrain and the segmented hindbrain, and for the elongation of the body axis (17, 18, 20). RA signalling has also been implicated in early heart patterning, forelimb induction, pancreas induction, lung induction, eye formation and some aspects of genitourinary tract development (2, 17, 18). However, our current understanding of the role of RA in adult stem cell populations, and tissue-specific progenitors is relatively limited.

We sought to investigate the role of RA signalling in regulating hepatic differentiation of HPCs. Using mouse liver tissues derived from E12.5 to postnatal days 28 (PD28), we found that the expression of RAR-\(\alpha\), RXR-\(\alpha\), RXR-\(\gamma\) were readily detected in most of the tested tissue samples. The expression of RAR-\(\beta\) and RXR-\(\beta\) was lower in perinatal samples and increased after birth, whereas RXR-\(\gamma\) was almost undetectable in prenatal tissues and increased after birth. Two of the three RA synthesis enzymes, Raldh1 and Raldh2 (retinal dehydrogenase 1 and 2) were expressed in all analysed tissues, while Raldh3 was weakly expressed in prenatal tissues but was readily detected in postnatal tissues. NCOR1 and NCOI2 were highly expressed in all analysed tissues, while the expression of NCOA1, NCOA2, NCOA3 and PCAF was decreased in perinatal tissues and increased after birth.

One of the major challenges in studying liver stem cell biology is the lack of stable HPCs. In order to further dissect the role of RA signalling in mouse liver development, we established reversible stable HPCs derived from the E14.5 mouse fetal liver through the retroviral integration of SV40 large T (25) (designated as HP14.5). The establishment of HPC lines from fetal liver should be useful for elucidation of the molecular signals required for specification, growth and differentiation of hepatoblasts and hepatocytes (7, 11, 13, 26, 27). The HP14.5 cells were shown to express high levels of early liver stem cell markers (e.g. Oct3/4, DLK and c-kit) but low levels of late liver markers (e.g. albumin and UGT1A). The expression of RARs, RXRs, NCORs, NCOAs and the Raldh isofoms was detected in HP14.5 cells. ATRA and/or 9CRA alone or as a synergistic pair were shown to effectively activate albumin promoter-driven reporter activity. We further demonstrated that upon ATRA and 9CRA treatment of HP14.5 cells the expression of early progenitor markers decreased and the expression of late/mature hepatocyte markers significantly increased. Lastly, ATRA and 9CRA were able to effectively induce glycogen synthesis/storage in HP14.5 cells, suggesting that RA may be capable of inducing the terminal differentiation of HPCs. Thus, our results have demonstrated that RA signalling may play an important role in regulating hepatic differentiation.

Materials and methods

Cell culture and chemicals

HEK-293 and Hepa1-6 lines were obtained from the ATCC (Manassas, VA, USA), and were maintained in complete Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin at 37°C in 5% carbon dioxide. ATRA and 9CRA were purchased from Biomol International (Plymouth Meeting, PA, USA). Unless indicated otherwise, all chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich (St Louis, MO, USA).

Isolation of mouse fetal hepatic progenitor cells

Primary mouse fetal HPCs were isolated from postcoitus day 14.5 (E14.5) mouse fetal liver. Briefly, the fetal liver was dissected, rinsed with sterile phosphate-buffered saline (PBS), and minced with a razor blade in 1.0 ml 0.25% trypsin and 1 mM EDTA. After 15-min incubation with gentle shaking at 37°C, complete DMEM was added to inactivate trypsin. Cells were plated on 100 mm dishes that were coated with type 1 collagen, and incubated at 37°C. Hepatocytes were isolated from the liver of 14-day-old mice in a similar fashion. All non-adherent cells were removed and the medium was changed every 3 days thereafter. The adherent cells were trypsinized and passed once for making stable cell lines.

Establishment of stable fetal hepatic progenitor cell lines

Primary liver cells isolated from E14.5 embryonic liver and 14-day-old mouse livers were infected with a retroviral vector SSR#69 that expresses SV40 large T antigen flanked with loxP sites, as described by Westerman and Leboulch (25). The infected cells were selected in 0.3 mg/ml hygromycin B (Invitrogen, Carlsbad, CA, USA) for 7–10 days. The stable cell pools were designated as HP14.5 and LC14D respectively. As a control line, the
mouse liver tumour line Hepa1-6 was infected with the same virus and selected with hygromycin B. Stable lines were scaled up and frozen in aliquots in liquid nitrogen tanks.

Construction of albumin promoter-driven Gaussia luciferase reporter

The 2.5 kb genomic DNA fragment upstream the exon 1 of mouse albumin gene was PCR amplified and subcloned into the BamHI/XhoI sites of our home-made retroviral reporter vector pSEB-GLuc to drive the expression of Gaussia luciferase, resulting in pSEB-Alb-GLuc. The reporter vector was used for transient transfection, as well as for making stable lines via retroviral infection. Authenticity of the PCR amplified fragments was verified by DNA sequencing. Cloning and construction details are available upon request.

Isolation of total RNA

For mouse liver tissues, freshly prepared mouse liver tissues were minced and homogenized in TRIZol Reagents (Invitrogen). For cultured cells, subconfluent cells were seeded in 75 cm² cell culture flasks or 100 mm dishes in a medium supplemented with 0.5% FBS. Total RNA was isolated using TRIZol Reagent according to the manufacturer’s instructions.

Reverse transcription and semiquantitative reverse transcriptase-polymerase chain reaction analysis

Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out as described (28–33). Briefly, 10 μg of total RNA were used to generate cDNA templates by reverse transcription with hexamer and Superscript II reverse transcriptase (Invitrogen). The first strand cDNA products were further diluted five- to 10-fold and used as PCR templates. The PCR primers were 18–20 mers, designed by using the PRIMER3 program, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, to amplify the 3’-end (approximately 120–150 bp) of the genes of interest (Supporting information Table S1). RT-PCR reaction was carried out by using a touchdown protocol: 94 °C for 1 min, followed by 25–30 cycles at 94 °C for 30 s, 56 °C for 1 min, and 70 °C for 1 min. RT-PCR products were resolved on 1.5% agarose gels. All samples were normalized by endogenous level of GAPDH.

Transfection and Gaussia luciferase reporter assay

Exponentially growing cells were seeded in 25 cm² cell culture flasks and transected with 2 μg per flask of pSEB-Alb-GLuc using LipofectAMINE (Invitrogen). At 16 h after transfection, cells were replated to 24-well plates and treated with ATRA, 9CRA or dimethyl sulfoxide (DMSO). Gaussia luciferase possesses a natural secretory signal and upon expression is secreted into the cell medium. Thus, at the indicated time points medium from the treated cells was collected for Gaussia luciferase assays using the Gaussia Luciferase Assay Kit (New England Biolabs, Ipswich, MA, USA). Each assay condition was performed in triplicate. Reporter activity was expressed as mean ± SD.

Immunofluorescence staining

Immunofluorescence staining was carried out as described (28, 30–33). Briefly, cells were fixed with methanol at −20 °C for 15 min and washed with PBS. The fixed cells were permeabilized with 1% NP-40 and blocked with 10% bovine serum albumin, followed by incubation with Oct3/4, DLK, c-kit, Alb or UGT1A antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 min. After washing, cells were incubated with DyLight 594-labelled secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 30 min. The presence of proteins of interest was examined under a fluorescence microscope. Stains without the primary antibody, or with control immunoglobulin G, were used as negative controls.

Periodic acid Schiff staining

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and then washed with ddH2O. The fixed cells were stained with 0.5% periodic acid solution for 5 min at room temperature, and rinsed with ddH₂O for 3 min. Cells were incubated with the Schiff’s reagents (Sigma-Aldrich) for 15 min at room temperature, washed with tap water for 3 min and then counterstained with haematoxylin solution for 2 min, followed by being thoroughly rinsed with tap water. Positive stain (purple red) was recorded using a microscope.

Histological evaluation

Freshly isolated mouse fetal liver and postnatal liver tissues were subjected to snap-freezing and sectioning. The slides were used for haematoxylin and eosine staining as previously described (29, 34–37).

Statistical analysis

We used the MICROSOFT EXCEL program to calculate standard deviations and statistically significant differences between samples using the two-tailed Student t test.

Results

Expression of liver marker genes at the different stages of hepatic development

To examine the potential role of retinoid signalling in the proliferation and differentiation of HPCs, we sought to examine the expression patterns of the components of retinoid signalling pathway in liver tissues derived from different stages of hepatic development. We first prepared
the freshly isolated liver samples from eight stages of mouse liver development, four prenatal and four postnatal stages ranging from E12.5 to 4 weeks after birth. Histology of three representative stages of liver samples is shown in Figure 1A. We set to isolate RNA from the liver samples and to confirm that the isolated liver tissues possessed the expression pattern of liver-related genes during mouse liver development. Using semiquantitative RT-PCR analysis, we determined the expression of hepatic marker genes (AFP, Alb and DLK), liver-related transcriptional factors (HNF-3α, HNF-3β and HNF-4α), and the liver late marker cytokeratin 18 (CK18). All samples were normalized with GAPDH. Polymerase chain reaction results were confirmed in at least three batches of independent experiments. AFP, α-foetoprotein; Alb, albumin; PD, postnatal days.

Expression of retinoid acid receptors, nuclear receptor co-activators and corepressors at the different stages of hepatic development

We determined the endogenous expression levels of the RAR and RXR isoforms in mouse liver samples. Using semiquantitative RT-PCR analysis, we demonstrated that the expression of RAR-γ, RXR-α and RXR-γ was readily detectable in all stages, whereas RAR-γ, RAR-β and RXR-β expressed at a lower level in prenatal liver tissues than that in postnatal samples (Fig. 2A). These results indicate that RAR and RXR receptors are in general widely expressed at different stages of mouse liver development. It is noteworthy that we initially conducted Western blotting analysis using antibodies against several RAR and RXR isotypes and observed their expression levels varied significantly from batch to batch of experiments (data not shown), possibly because of the low antibody affinities and/or the less than ideal quality of the protein samples prepared from liver tissues. Furthermore, the expression level of different genes may not be compared by Western blotting because of the varied affinities and reactivities of different antibodies. Thus, we used RT-PCR to determine the relative expression of RAR and RXR isotypes.

Retinoic acid is produced solely from vitamin A derivative Rald by Raldh. We examined the expression of the three Raldh isoforms. As shown in Figure 2B, expression of Raldh1 and Raldh2 were readily detectable in all tissues, while Raldh3 had much lower expression, particularly in prenatal tissues. Like other members of the nuclear receptor superfamily, RAR and RXR activities are regulated by a network of nuclear receptor corepressors (e.g. NCOR1 and NCOR2), NCOAs (e.g. NCOA1, NCOA2 and NCOA3), receptor-interacting protein 140 (RIP140), and/or histone acetyltransferase PCAF. We examined the endogenous expression of these factors in the liver tissues. As shown in Figure 2C, both NCOR1 and NCOR2 expressed in all liver samples. However, NCOA1, NCOA2 and NCOA3 tended to have a weaker expression in perinatal liver tissues (e.g. in E16.5 and E18.5), and to
regain their expression after birth (Fig. 2C). RIP140 and PCAF exhibited a similar expression trend to that of the NCOAs' except their expression level was higher. These results indicate that RAR and RXR activities may be repressed in perinatal liver development.

Establishment and characterization of fetal liver progenitor lines

While the above studies using the freshly isolated liver tissues provide insightful information about the potential role of RA signalling components in hepatic differentiation, stable progenitor cell lines are essential for dissecting the role of RA signalling in regulating the proliferation and differentiation of HPCs. In order to establish such stable progenitor lines, we used the previously reported reversible immortalization vector SSR #69 (25), which uses the overexpression of SV40 large T antigen flanked by loxP sites (Fig. 3A). Thus, the expression of large T can be inactivated by introducing Cre recombinase. Using the primary fetal hepatocytes isolated from E14.5 mouse embryo fetal liver and primary hepatocytes from 2 weeks mouse liver tissues, we infected the cells with a retrovirus packaged SSR #69 and selected for hygromycin-resistant stable cell pools (25), designated as HP14.5 and LC14D. When the stable cells were infected with Ad-Cre, we were able to detect the loss of large T antigen expression via Western blotting (data not shown), indicating that the integration of large T antigen is reversible. The immortalized stable cells retained hepatocyte-like phenotype when grown into confluence (Fig. 3B), in comparison with that of mouse liver tumour line Hepa1-6.

We further examined the expression of hepatic progenitor markers and mature hepatocyte markers using the immunofluorescence staining. As shown in Figure 3C, pluripotent progenitor cells marker Oct3/4 and early hepatic marker DLK were readily detected in HP14.5 cells and marginally detectable in LC14D and Hepa1-6 cells. Albumin, as a liver-specific marker, was detectable in all three lines, whereas Hepa1-6 cells had the highest level of expression. The mature hepatocyte marker UGT1A was expressed at a higher level in LC14D and Hepa1-6 cells than that in HP14.5 cells. Interestingly, four of the five proteins (DLK, c-kit, Alb and UGT1A) examined by immunofluorescence staining are not nuclear proteins but appear to have nucleus and/or whole cell staining. Although we do not have any explanation for this staining pattern, there are numerous reports about the whole cell/nuclear staining patterns for these proteins (38–41). Other early hepatic markers Thy1, CD34 and N-CAM were highly expressed in HP14.5 cells, while the mature hepatic marker ApoB was undetectable in HP14.5 cells (data not shown). Taken together, these results suggest that the immortalized stable HP14.5 cells retain many if not all of the HPC phenotype.

Expression patterns of the retinoic acid signalling components in the stable liver progenitor lines

We next sought to determine the expression patterns of RA signalling components in the stable HPCs. As shown...
in Figure 4A, expression of RXR-α, RXR-β, RAR-α and RAR-γ was readily detected in HP14.5 cells, as well as in LC14D and Hepa1-6. However, RXR-γ (and to a lesser extent, RAR-β) weakly expressed in HP14.5 and LC14D cells. On the other hand, expression of all NCORe and NCOAe was readily detected in HP14.5 cells (Fig. 4B). Similarly, PCAF, RIP140 and the three Raldh genes were expressed in HP14.5 cells. We also found that the expression of cellular retinoic acid-binding protein (CRABP), which is involved in retinol metabolism, was undetectable in HP14.5 and LC14D cells, but highly expressed in Hepa1-6 cells (Fig. 4B). It is noteworthy that the expression of Raldh3 in HP14.5 cells was higher than that detected in E14.5 liver tissue (Fig. 2B). Although the exact causes of the discrepancy are not known, two possibilities may be partially accountable for the expression differences. Firstly, the in vitro culture conditions may affect the gene expression profile of HP14.5 cells. Secondly, the freshly isolated liver tissues may contain non-hepatocyte components (such as liver stellate cells, Fig. 3. Establishment and characterization of stable hepatic progenitor cells. (A) Schematic representation of the previously used retroviral vector SSR#69 for reversible immortalization of primary cells (25). (B) Morphology of the stable fetal hepatic cells HP14.5. Cultured HP14.5, LC14D and Hepa1-6 were stained with haematoxylin and eosine. Magnification, ×200. (C) Immunofluorescence staining of stem cell markers and mature hepatocyte markers in HP14.5, LC14D and Hepa1-6 cells. Subconfluent cells were fixed and probed with antibodies against Oct4, DLK, c-kit, Alb and UGT1A (Santa Cruz Biotechnology), followed by staining with fluorescein isothiocyanate or Texas red-labelled secondary antibodies. Cells stained without primary antibodies were used controls. Magnification, ×200.
Kupffer cells, parenchymal cells and sinusoidal endothelial cells) and thus the expression pattern was a reflection of the mixed cell types. Nonetheless, our results indicate that HP14.5 cells possess the essential features of HPCs and exhibit a similar if not identical expression pattern of the RA signalling components to that of the freshly isolated liver tissues of the early stages of liver development.

Retinoic acid effectively induces hepatic differentiation of hepatic progenitor cells

We next sought to determine the role of RA in hepatic differentiation. In order to quantitatively assess RA-induced hepatic differentiation, we constructed a mouse Alb-GLuc reporter (Fig. 5A). When this reporter was transiently introduced into HP14.5 and Hepa1-6 cells, we found that both ATRA (1 μM) and 9CRA (1 μM) effectively induced Gaussia luciferase (GLuc) activity at day 5 and day 9 (Fig. 5B). Differences in GLuc activity between ATRA and 9CRA treated HP14.5 cells were not statistically significant. Although ATRA and 9CRA were also shown to induce GLuc activity in liver tumour line Hepa1-6 at day 9, the magnitude of activation was only about 20% of that in HP14.5 cells, suggesting that, unlike HPCs, liver tumour cells have limited capacity of differentiation. It is noteworthy that when HP14.5 cells were transfected with Alb-GLuc reporter plasmid and infected with AdGFP or AdCre in the presence of ATRA or 9CRA, GLuc activity was assayed at day 5, day 7 and day 9 after treatment and that the Alb-GLuc activity was not affected by Cre expression in HP14.5 cells (data not shown).

We further tested whether ATRA and 9CRA acted synergistically on hepatic induction. We found that ATRA induced GLuc activity in HP14.5 cells in a dosage and time-dependent fashion, although the dosage and time were shown plateaus at 1 μM and 8 days respectively (Fig. 6A). Similarly, 9CRA activity was peaked at 1 μM and 11 days respectively (Fig. 6B). It seems that ATRA is more effective than 9CRA at the same concentrations. Furthermore, we found that suboptimal concentrations of 9CRA...
Retinoic acid (RA) induction of albumin promoter-driven Gaussia luciferase (Alb-GLuc) reporter activity in hepatic progenitor cells. (A) Construction of the Alb-GLuc reporter. An Alb-GLuc reporter is schematically shown. (B) RA induction of Alb-GLuc activity in HP14.5 cells. HP14.5 and Hepa1-6 cells were transfected with Alb-GLuc reporter plasmid and treated with all-trans-retinoic acid (ATRA) or 9-cis-retinoic acid (9CRA) (1.0 μM). Relative Gaussia luciferase activity (mean ± SD) was assayed at the indicated time points. Differences between ATRA and 9CRA treated samples were statistically insignificant (P < 0.10). Each assay condition was carried out in triplicate.

Retinoic acid induces terminal differentiation of hepatic progenitor cells

We analysed the hepatic markers that indicate different stages of hepatic differentiation upon ATRA and 9CRA stimulation. Using RT-PCR, we demonstrated that early marker DLK decreased after prolonged treatment of ATRA and 9CRA although AFP expression did not change significantly (Fig. 7A). CK19 serves as a bipoten-

tial stem cell marker for both hepatic stem cells and biliary epithelial progenitors (3, 8). But CK19 expression decreases in differentiated hepatocytes while its expression increases in pancreatic islet cells and biliary ductal cells. As shown in Figure 7A, CK19 expression decreased significantly after 6- or 9-day treatment of ATRA and 9CRA, while mature hepatocyte marker CK18 (3, 8) increased after ATRA and 9CRA treatment (Fig. 7A). Immunofluorescence staining revealed that expression of Alb and mature hepatocyte marker UDP-glucuronosyl transferase (UGT1A) was significantly induced after 9-day treatment of HP14.5 with ATRA and 9CRA, while expression of the early marker Oct3/4 was reduced upon ATRA and 9CRA treatment (Fig. 7B).

Lastly, we tested the effect of RA on the terminal differentiation of HPCs by using Periodic acid Schiff (PAS) staining method. PAS staining is primarily used to identify glycogen in tissues although glycoprotein and proteoglycans can also be positively stained. We treated HP14.5 cells with ATRA (1.0 μM), 9CRA (1.0 μM) or DMSO for 10 days. When the cells were fixed and subjected to PAS staining, positive stains were significantly increased in RA-treated HP14.5 cells (Fig. 7C). Taken together, we have demonstrated that activation of RA signalling pathway can effectively induce the terminal differentiation of HPCs.

Discussion

In this report, we investigated the role of RA signalling in regulating hepatic differentiation of HPCs. Using mouse liver tissues derived from E12.5 to PD28, we found that the expression of RAR-α, RXR-α and RXR-γ were readily detected in most of the tested tissue samples. The expression of RAR-β and RXR-β was lower in perinatal samples and increased after birth, whereas RAR-γ was almost undetectable in prenatal tissues and increased after birth. Two of the three RA synthesis enzymes, Raldh1 and Raldh2 were expressed in all analysed tissues, while Raldh3 was weakly expressed in prenatal tissues but was readily detected in postnatal tissues. The NCOR1 and NCO2 were highly expressed in all analysed tissues, while the expression of NCOA1, NCOA2, NCOA3 and PCAF was decreased in perinatal tissues and increased after birth. Using the reversibly immortalized HPC14.5 cells derived from the E14.5 mouse fetal liver (25), we showed that the HP14.5 cells expressed high levels of early liver stem cell markers but low levels of late liver markers. Expression of RARs, RXRs, NCOs and NCOAs and the Raldh isoforms was readily detected in HP14.5 cells. ATRA and/or 9CRA alone or as a synergistic pair were shown to effectively activate albumin promoter-driven reporter activity. We further demonstrated that upon ATRA and 9CRA treatment of HP14.5 cells the expression of early progenitor markers decreased and the expression of late/mature hepatocyte markers significantly increased. Lastly, ATRA and 9CRA were able to effectively induce glycogen synthesis/storage in HP14.5
cells, suggesting that RA may be capable of inducing the terminal differentiation of HPCs. Thus, our results have demonstrated that RA signalling may play an important role in regulating hepatic differentiation.

The establishment of HPC lines from fetal liver should be useful for elucidation of the molecular signals required for specification, growth and differentiation of hepatoblasts and hepatocytes (7, 11, 13, 26, 27). Normal liver is proliferatively quiescent; but hepatocyte loss through partial hepatectomy, viral infection or inflammation, invokes a rapid regenerative response from all cell types in the liver to restore liver mass. Liver transplantation is currently the only therapeutic option for patients with terminal chronic liver diseases or severe acute liver failure. Although the functional role of liver stem cells in liver regeneration remains controversial (3), HPCs are a promising source for liver repopulation after cell transplantation (5–8, 42). Mesenchymal stem cells and embryonic stem cells can be induced to differentiate along the hepatic lineage in culture, but these cells are currently inefficient in repopulating the liver (5–8). Thus, as for any other stem cell-based therapies, the challenge is to understand the molecular mechanisms underlying hepatic differentiation and to identify factors, such as RA signalling pathway, which can effectively induce and direct HPCs to differentiate into mature hepatocytes.

Retinoic acid plays an important role in tissue patterning and morphogenesis during development (2, 17, 18, 20). RARs (RAR-α, RAR-β and RAR-γ isotypes) bind ATRA and 9CRA, and the retinoid receptors (RXR-α, RXR-β and RXR-γ isotypes) bind 9CRA only (20). For each RAR isotype, several isoforms harbouring distinct N-terminal domains arise from the differential usage of two promoters and alternative splicing. There are two major isoforms for RAR-α (α1 and α2) and for RAR-γ (γ1 and γ2) and four major isoforms for RAR-β (β1–β4). Similarly, at least two isoforms have been identified for RXR-α (α1 and α2), RXR-β (β1 and β2) and RXR-γ (γ1 and γ2) (20). Rara-, Rarb- and Rarg-null mutant mice are viable, but they display some aspects of the fetal and postnatal vitamin A deficiency (VAD) syndromes, as well as few additional congenital malformations (20). Mutants lacking two RAR isotypes (Rara/b-, Rara/g- and Rarb/g-null mutants) die in utero or at birth from severe developmental defects that include the complete spectrum of malformations belonging to the fetal VAD-induced syndrome (20). Numerous compound mutants among RXRs, RARs and RA synthesizing enzymes (Raldh1, Raldh2 and
Raldh3) have revealed an extensive functional redundancy within the members of each family (RARs or RXRs) although each of these members appears to individually exert at least one specific physiological function (20).

Consistent with our conclusion, it has been shown that Wt1 and RA signalling are essential for stellate cell development and liver morphogenesis (43). In the absence of Wt1, the liver is reduced in size, and shows lobing abnormalities. Coelomic cells lining the liver of Wt1-null embryos show decreased or absent Raldh2 expression, the population of cells expressing high levels of RXR-α is much reduced and the proliferation of hepatoblasts and RXR-α-positive cells is significantly decreased. It was similar to that was described a similar retardation of liver growth in RXR-α-null mice (44), as well as in chick embryos after inhibition of RA synthesis.

Fig. 7. Retinoic acid (RA) induced terminal differentiation of hepatic progenitor cells. (A) HP14.5 cells were treated with all-trans-retinoic acid (ATRA) or 9-cis-retinoic acid (9CRA) (1 μM), or dimethyl sulfoxide (DMSO). RNA was isolated from the treated cells at the indicated time points and subjected to reverse transcriptase-polymerase chain reaction analysis (see ‘Materials and methods’). Expression of α-foetoprotein (AFP), DLK, cytokeratin (CK)18 and CK19 was analysed. All samples were normalized with GAPDH. (B) RA-induced expression of late hepatic markers. HP14.5 cells were treated with ATRA or 9CRA (1 μM), or DMSO for 9 days. Cells were fixed and stained with Oct3/4, Alb and UGT1A antibodies, followed by staining with DyLight 594-labelled secondary antibodies. Fluorescence signals were recorded with a fluorescent microscope. (C) RA induced terminal differentiation of hepatic progenitor cells. HP14.5 cells were treated ATRA or 9CRA (1.0 μM) or DMSO for 10 days. Cells were fixed and subjected to Periodic acid Schiff staining. Positive stains (purple red) are indicated by arrows. The staining results were confirmed by at least three batches of independent experiments; and representative results are shown.
Thus, Wt1 expression in cells delaminating from the coelomic epithelium is essential for the expansion of the progenitor population of liver stellate cells and for liver morphogenesis that is mechanistically at least in part mediated via the RA signalling pathway (43).

Currently, our understanding about RA signalling in liver development and hepatic regeneration is relatively limited. Strategies for spatio-temporally controlled mutagenesis of RARs and RXRs in mouse liver have yet to be carried out. It is conceivable that the RAR/RXR transcription complex may activate or interact with the critical genes that control hepatocyte differentiation. Indeed, it was shown that HNF-3α is activated upon RA-induced differentiation of mouse F9 embryonal carcinoma cells (46). The bona fide RA response element has been found in HNF-4α and HNF-4γ is upregulated in vivo upon RA signalling (47). Further, HNF-3α transcription factor is a primary target for RA action (48). It has been reported that retinoids regulate the differentiated phenotype of rat hepatic lipocytes in primary culture (49). In an early study, it was shown that expression of RAR-α was significantly elevated in vitamin A-deficient livers following partial hepatectomy, whereas in the vitamin A-supplemented animals RAR-β expression was the major species after partial hepatectomy (50). A recent study demonstrated that retinoids induce cytochrome p450 3A4 through RXR-α and vitamin D receptor-mediated pathway (51). Thus, the functional roles of RARs and RXRs in regulating hepatic differentiation of stem cells and/or HPCs remain to be thoroughly investigated. Interestingly, it has been reported that ATRA increased DNA synthesis, which was not because of ATRA-induced liver damage and regeneration, but rather the consequence of a direct mitogenic effect (52).

The ability of RA to promote hepatic differentiation may be exploited as chemotherapeutic and/or chemopreventive measures for liver cancer (53–62). Interestingly, it has been reported that exogenous RA may be teratogenic, depending on the dose and on the developmental stage at the time of exposure (63, 64). A chromosomal translocation produces a chimeric protein between RAR-α and promyelocyte leukemia protein (PML) (65). PML–RAR-α fusion protein works as a dominant negative receptor in the leukemic cells. PML–RAR-α fusion protein interferes with the normal function of RAR-α and/or PML, and in turn blocks cell maturation at the stage of promyelocytes (65). Numerous studies have shown that retinoids can inhibit or reverse the carcinogenic process of haematological malignancy, as well as premalignant and malignant lesions in the oral cavity, head and neck, breast, skin, bone and liver (58, 66–68). It is conceivable that understanding the molecular mechanisms of retinoid-regulated hepatic differentiation of HPCs may expand our knowledge about hepatocellular carcinogenesis and its potential treatment.

In summary, we have demonstrated that most components of the RA signalling pathways are expressed in the developing stages of mouse livers, and that ATRA and 9CRA can effectively induce terminal hepatic differentiation of mouse fetal liver-derived HPCs. We are currently characterizing individual clones for their progenitor properties. Future studies should be directed to understand the molecular basis of RA-induced hepatic differentiation, which may help to unravel the pathogenesis of some liver diseases and/or lead to the development of retinoids as potential therapeutic or preventive agents for liver cancer.

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References


Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of RT-PCR Primers.

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