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Wnt Antagonist SFRP3 Inhibits the Differentiation of Mouse Hepatic Progenitor Cells

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ABSTRACT

Wnt/β-catenin pathway plays an important role in regulating embryonic development. Hepatocytes differentiate from endoderm during development. Hepatic progenitor cells (HPCs) have been isolated from fetal liver and extrahepatic tissues. Most current studies in liver development and hepatic differentiation have been focused on Wnts, β-catenin, and their receptors. Here, we sought to determine the role of Wnt antagonists in regulating hepatic differentiation of fetal liver-derived HPCs. Using mouse liver tissues derived from embryonic day E12.5 to postnatal day (PD) 28, we found that 13 of the 19 *Wnt* genes and almost all of Wnt receptors/co-receptors were expressed in most stages. However, Wnt antagonists SFRP2, SFRP3, and Dkk2 were only detected in the early stages. We established and characterized the reversible stable HPCs derived from E14.5 mouse fetal liver (HP14.5). HP14.5 cells were shown to express high levels of early liver progenitor cell markers, but low levels or none of late liver markers. HP14.5 cells were shown to differentiate into mature hepatocytes upon dexamethasone (Dex) stimulation. Dex-induced late marker expression and albumin promoter activity in HP14.5 cells were inhibited by exogenous expression of SFRP3. Furthermore, Dex-induced glycogen synthesis of PAS-positive HP14.5 cells was significantly inhibited by SFRP3. Therefore, our results have demonstrated that the expression of Wnt antagonists decreases as hepatic differentiation progresses, suggesting that a balanced Wnt signaling may be critical during mouse liver development and hepatic differentiation. J. Cell. Biochem. 108: 295–303, 2009.

KEY WORDS: WNT/β-CATENIN SIGNALING; SFRPs; SFRP3; HEPATIC PROGENITOR CELLS; HEPATIC DIFFERENTIATION; LIVER REGENERATION

Wnt proteins are a large family of secreted cysteine-rich glycoproteins that act as short-range ligands to locally activate receptor-mediated signaling cascades. Wnts play important roles in cell fate specification, tissue patterning, and control of asymmetric cell division, although Wnt signaling in mammals is more complex [Luo et al., 2007a; Wodarz and Nusse, 1998]. Wnts

Abbreviations used: AFP; α-fetoprotein; Alb; albumin; APC; adenomatous polyposis coli; Dex; dexamethasone; DLK; Delta-like protein; Fzd; the frizzled receptor; GFP; green fluorescent protein; HNFs; hepatic nuclear factors; HPCs; hepatic progenitor cells; LRP; LDL-receptor related protein; PAS; Periodic acid-Schiff staining; RT-PCR; reverse transcriptase-PCR; SFRPs; soluble Fzd-related proteins.

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Yang Bi and Jiayi Huang contributed equally to the work.

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activate at least three signaling pathways and the best understood one is the canonical Wnt pathway that activates transcriptional activity of β-catenin/T cell factor (Tcf) [Wodarz and Nusse, 1998; Luo et al., 2007a]. By contrast, non-canonical Wnts seem to act through β-catenin/Tcf-independent pathways that regulate cell movement and organization [Wodarz and Nusse, 1998]. Abnormal Wnt signaling has been associated with many human diseases [Luu et al., 2004; Clevers, 2006; Luo et al., 2007a].

Free cytoplasmic β-catenin is rapidly degraded through proteosome promoted by APC/Axin/GSK3β complex [Wodarz and Nusse, 1998; Luo et al., 2007a]. Wnts bind to frizzled (Fzd) receptors and LDL-receptor related protein (LRP) 5/6 co-receptors. Soluble Fzd-related proteins (SFRPs) act as antagonists by squelching Wnts, while Dkk proteins sterically hinder Wnt interaction with LRP5/6. Upon Wnt binding, Axin translocates to cell membrane and interacts with LRP5/6, and Wnt binding to Fzd results in hyperphosphorylation of disheveled (Dvl) by Casein kinases (CK) Iε and II, preventing GSK3β from phosphorylating β-catenin. Unphosphorylated β-catenin is stabilized by escaping the recognition of by β-TrCP and translocates to nucleus where it engages LEF/Tcf to regulate expression of downstream targets [He et al., 1998a, 1999; Clevers, 2006].

Hepatocytes differentiate from endoderm during development [Zaret, 2008; Zaret and Grompe, 2008]. Wnt/β-catenin pathway plays an important role in embryonic liver development [Thompson and Monga, 2007; Zaret, 2008; Zaret and Grompe, 2008]. β-Catenin expresses high levels during E10–E14, and is localized in nucleus/cytoplasm of hepatoblasts [Micsenyi et al., 2004], indicating that β-catenin activation is critical for early postnatal liver growth [Apte et al., 2007; Thompson and Monga, 2007]. Conditional deletion of *β-catenin* in hepatoblasts disrupts hepatic morphogenesis and survival during mouse development [Tan et al., 2008]. Overexpression of constitutively active β-catenin in developing liver led to an increase in liver size and an expansion of hepatocyte precursor cell population [Cadoret et al., 2001; Tan et al., 2005]. Furthermore, β-catenin protein is rapidly but transiently increased within minutes of hepatectomy [Monga et al., 2001]. Wnt/β-catenin signaling is active in mouse hepatic transit amplifying progenitor cells [Hu et al., 2007]. Accordingly, most Wnts and Fzds were shown to express in the adult mouse liver along with individual cell types [Zeng et al., 2007].

Current studies on Wnt signaling in liver development are focused on Wnts, β-catenin, and their receptors [Thompson and Monga, 2007; Hay et al., 2008; Zaret, 2008]. Here, we investigate the role of Wnt antagonists in hepatic differentiation. Using mouse liver tissues derived from embryonic day 12.5 (E12.5) to postnatal days 28 (PD28), we found that 13 of the 19 Wnt, all Wnt receptors and co-receptors were expressed in the tested liver tissues. However, Wnt antagonists SFRP2, SFRP3 (aka., FrzB), and Dkk2 only expressed in the early stages of liver development. We further established reversible stable hepatic progenitor cells (HPCs) derived from E14.5 mouse fetal liver (designated as HP14.5) [Westerman and Leboulch, 1996]. The stable HPCs expressed high levels of early liver stem cell markers but low levels of late liver markers. HP14.5 cells differentiated into mature hepatocytes upon dexamethasone (Dex) stimulation, which was inhibited by Wnt antagonist SFRP3. Thus, our results suggest that a tight regulation of Wnt signaling activity may be critical to mouse liver development.

MATERIALS AND METHODS

CELL CULTURE AND CHEMICALS

HEK-293 and Hepa1–6 lines were obtained from ATCC (Manassas, VA), and were maintained in complete DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. Unless indicated otherwise, all chemicals were purchased from Fisher or Sigma-Aldrich.

ISOLATION OF MOUSE FETAL HEPATIC PROGENITOR CELLS (HPCs)

Primary fetal hepatic progenitors were isolated from E14.5 mouse embryos. Briefly, the fetal liver was dissected and rinsed with sterile PBS, and minced with a razor blade in 1 ml 0.25% trypsin/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 precoated with type I collagen. Two-week hepatocytes were isolated from 14-day-old mouse liver in a similar fashion. Non-adherent cells were removed. Medium was changed every 3 days. Adherent cells were trypsinized and passaged once for making stable cell lines.

ESTABLISHMENT OF STABLE HPC 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 [Westerman and Leboulch, 1996]. Cells were selected with 0.3 mg/ml hygromycin B (Invitrogen) for 7–10 days. Stable cell pools were designated as HP14.5 and LC14D, respectively. As a control line, mouse liver tumor line Hepa1–6 was infected with the same virus and selected with hygromycin B. The stable lines were scaled up and frozen in aliquots in liquid nitrogen tanks.

CONSTRUCTION OF ADENOVIRUSES EXPRESSING CRE RECOMBINASE, SFRP3, AND GFP

Recombinant adenoviruses expressing Cre and SFRP3 were generated using AdEasy technology as described [He et al., 1998b, 1999; Cheng et al., 2003; Kang et al., 2004; Luo et al., 2007b; Tang et al., 2008; Sharff et al., 2009]. Briefly, coding regions of Cre and SFRP3 were PCR amplified, subcloned into the pAdTrack-TO4, and used to generate adenoviral recombinants. Ad-Cre and Ad-SFRP3 were produced and amplified in HEK-293 as described [He et al., 1998b, 1999; Luo et al., 2007b; Tang et al., 2008]. An analogous Ad-GFP was used as a control [He et al., 1998b, 1999; Luo et al., 2007b].

CONSTRUCTION OF ALBUMIN PROMOTER-DRIVEN GAUSSIA LUCIFERASE (Alb-GLuc) REPORTER

Genomic DNA fragment (2.5 kb) upstream exon 1 of mouse albumin (Alb) gene was PCR amplified and subcloned into a homemade pSEB-GLuc retroviral vector to drive expression of Gaussia luciferase (pSEB-Alb-GLuc). The reporter vector can be used for transient transfection, and for making stable lines. Authenticity of the PCR amplified fragments was verified by DNA sequencing.

ISOLATION OF TOTAL RNA AND RT-PCR

Freshly prepared mouse liver tissues were minced and homogenized in TRIZol Reagents (Invitrogen). For cultured cells, subconfluent cells were seeded in 75-cm^2 cell culture flasks or 100 mm dishes with 0.5% FBS DMEM. Total RNA was isolated using TRIZol Reagent. Reverse transcriptase-PCR (RT-PCR) was performed as described [Peng et al., 2003; Luo et al., 2004; Si et al., 2006; Tang et al., 2008]. Primers were 18–20mers, designed by using *Primer3* program to amplify the 3′-end of the gene of interest (Supplemental Table I). PCR reaction was performed by using touchdown protocol as described [Peng et al., 2003; Luo et al., 2004; Si et al., 2006; Tang et al., 2008]. PCR products were resolved on agarose gels. Samples were normalized by endogenous GAPDH.

TRANSFECTION AND LUCIFERASE REPORTER ASSAY

Cells were seeded in 25 cm^2 flasks and transfected pSEB-Alb-GLuc using LipofectAMINE (Invitrogen). At 16 h after transfection, cells were replated to 24-well plates and infected with Ad-SFRP3 or Ad-GFP, and/or treated with Dex (1 μM). Gaussia luciferase has a secretory signal and is secreted into cell medium. Medium from treated cells was collected for Gaussia luciferase assays using the Gaussia Luciferase Assay Kit (New England Biolabs). Each assay condition was performed in triplicate. Reporter activity was expressed as mean ± SD.

WESTERN BLOTTING

Western blotting was performed as previously described [Peng et al., 2003; Luo et al., 2004; Si et al., 2006; Tang et al., 2008]. Briefly, cells were collected and lysed in Laemmli buffer. Cleared total cell lysate was denatured by boiling and loaded onto a 4–20% gradient SDS–PAGE. After electrophoretic separation, proteins were transferred to an Immobilon-P membrane. Membrane was blocked with SuperBlock Blocking Buffer, and probed with the primary antibody, anti-SV40 large T antigen, anti-AFP, anti-UGT1A, or anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with a secondary antibody conjugated with horseradish peroxidase. The proteins of interest were detected by using SuperSignal West Pico Chemiluminescent Substrate kit.

IMMUNOFLUORESCENCE STAINING

Immunofluorescence staining was performed as described [Peng et al., 2003; Luo et al., 2004; Si et al., 2006; Tang et al., 2008]. Briefly, cells were fixed with methanol, permeabilized with 1% NP-40, and blocked with 10% BSA, followed by incubating with Oct4, Delta-like protein (DLK), Alb, or UGT1A antibody (Santa Cruz Biotechnology) for 60 min. After washing, cells were incubated with FITC or Texas Red-labeled secondary antibody (Santa Cruz Biotechnology) for 30 min. Stains were examined under a fluorescence microscope. Stains without primary antibodies, or with control IgG, were used as negative controls.

PERIODIC ACID-SCHIFF (PAS) STAINING

Cells were fixed with 4% paraformaldehyde, washed with ddH_2O, and stained with 0.5% periodic acid solution. Cells were then incubated with Schiff's reagents, washed with tap water, and then counterstained with hematoxylin solution, followed by being thoroughly rinsed with tap water. Positive stain (purple red) was recorded under a microscope.

STATISTICAL ANALYSIS

Microsoft Excel was used to calculate standard deviations (SD) and statistically significant differences between samples using the two-tailed Student's *t*-test.

RESULTS

EXPRESSION OF LIVER MARKERS DURING HEPATIC DEVELOPMENT

To examine the expression patterns of the components of Wnt signaling pathway in liver tissues derived from different stages of hepatic development, we isolated RNA samples from nine stages of liver development, four prenatal and five postnatal stages ranging from E12.5 to 4 weeks after birth. Using semi-quantitative PCR analysis, we determined the expression of hepatic marker genes, liver-related transcriptional factors, and liver late marker CK18 (as well as the biliary epithelial marker CK19). As shown in Figure 1, expression of liver-specific genes such as α-fetoprotein (AFP) and Alb was readily detected in all stages of liver samples, while the early hepatic marker DLK exhibited a higher expression in the prenatal samples. Although expression levels varied, the hepatic nuclear factors HNF3α, HNF3β, and HNF4α were expressed in all samples. Cytokeratin 19 (CK19) serves as a bipotential stem cell marker for both hepatic stem cells and biliary epithelial progenitors [Walkup and Gerber, 2006; Zaret and Grompe, 2008]. But CK19 expression decreases in differentiated hepatocytes while its expression

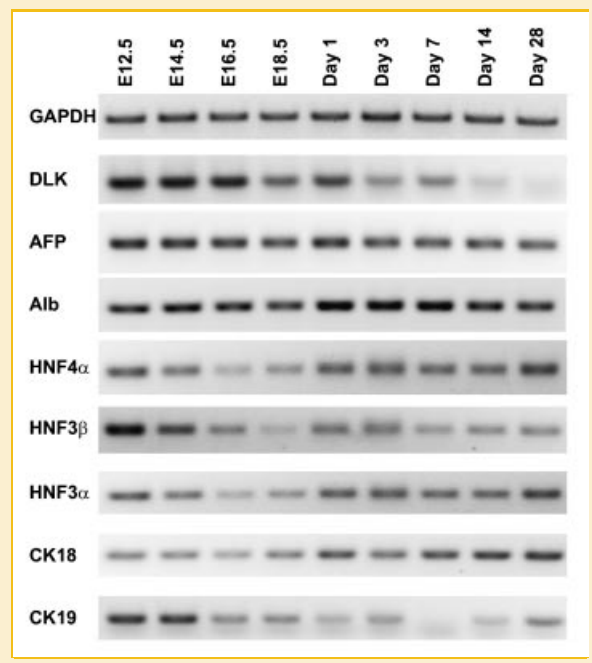

Fig. 1. Expression of liver-related genes during mouse liver development. Total RNA was isolated from mouse liver tissues derived from nine stages of liver development. All samples were normalized with GAPDH. RT-PCR results were confirmed in three batches of independent experiments.

increases in pancreatic islet cells and biliary ductal cells. As shown in Figure 1, CK19 expression decreased in postnatal liver tissues, while mature hepatocyte marker CK18 increased after birth [Walkup and Gerber, 2006; Zaret and Grompe, 2008]. Thus, these results indicate that RNA isolated from the different stages of liver tissues represents the respective stages of liver development.

EXPRESSION OF THE 19 Wnts AT THE DIFFERENT STAGES OF HEPATIC DEVELOPMENT

We next sought to determine the endogenous expression levels of the 19 Wnt genes identified so far in mice. Using semi-quantitative RT-PCR analysis, we demonstrated that the expression of 13 of the 19 *Wnt* genes was detectable (Fig. 2). These include Wnt1, 2, 2B, 3, 3A, 4, 5A, 6, 8A, 9B, 10A, 10B, and 16. Among them, Wnt2B, 3A, 4, 8A, 9B, 10A, and 10B were highly expressed at all of the tested stages of liver development. It is noteworthy that Wnt2 and 5A expressed at the later stages (e.g., 1 week after birth), while Wnt6 and 16 expressed at the early stage (e.g., before E14.5) of liver development (Fig. 2). These results suggest that Wnt family members may exert a diverse effect on hepatic differentiation.

Fig. 2. Expression of the 19 Wnts during mouse liver development. The same set of samples as that described in Figure 1 was used for RT-PCR analysis of 19 Wnt genes. All samples were normalized with GAPDH as shown in Figure 1. PCR results were confirmed in three batches of independent experiments.

EXPRESSION OF THE Wnt RECEPTORS AND CO-RECEPTORS DURING HEPATIC DEVELOPMENT

Wnt proteins execute their diverse biological functions through interaction with their cognate receptors Fzd and co-receptors LRP5/6. Ten Fzd receptors have been identified [Luo et al., 2007a; Thompson and Monga, 2007]. As shown in Figure 3A, all ten Fzd receptors were expressed in the isolated liver tissues, while the expression of Fzd2, 3, 5, 6, 7, and 8 slightly decreased during liver development. Fzd4 and 10 increased their expression postnatally. The expression of both LRP5 and 6 was readily detected, although the expression of LRP6 increased after birth (Fig. 3B). These results indicate that the expression of Wnt receptors and co-receptors is rather ubiquitous and abundant during liver development.

DISTINCT EXPRESSION PATTERNS OF Wnt ANTAGONISTS DURING HEPATIC DEVELOPMENT

As Wnt signaling pathway plays an important role in regulating stem cell fate [Clevers, 2006; Luo et al., 2007a], we postulated that a tight regulation of Wnt signaling activity by Wnt antagonists may be important for the functions of Wnts in hepatic differentiation. Wnt antagonists are divided into two categories: soluble frizzled-related proteins (SFRPs) that compete with Wnt ligands for binding to Fzd receptors, and Dickkopf proteins (Dkks) that bind to LRP co-receptors [Wodarz and Nusse, 1998; Miller, 2002; Thompson and

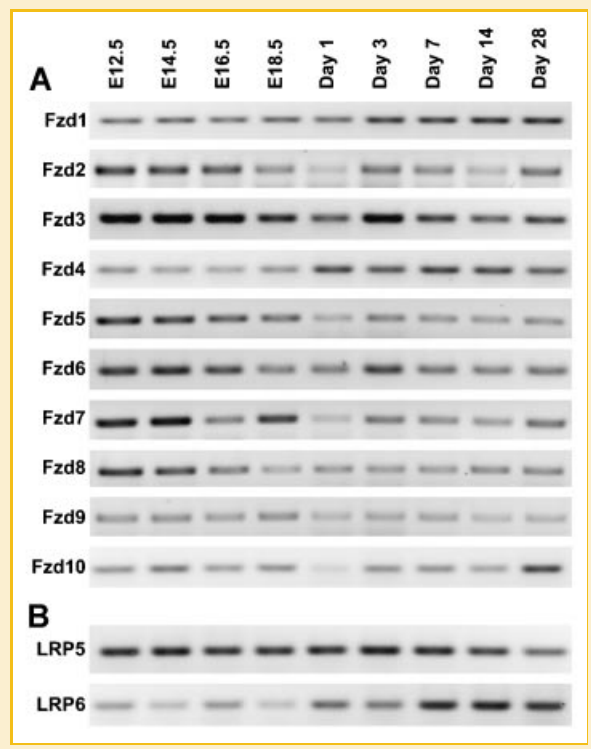

Fig. 3. Expression of Wnt receptors and co-receptors during mouse liver development. The same sample set as described in Figure 1 was used for RT-PCR. A: RT-PCR analysis of 10 Fzd receptor expression. B: RT-PCR analysis of Wnt co-receptor LRP5 and LRP6 expression. PCR results were confirmed in three batches of independent experiments. All samples were normalized with GAPDH as shown in Figure 1.

Monga, 2007]. There have been five SFRPs and three Dkks identified in mice. Through RT-PCR analysis, we demonstrated that SFRP1, 4, and 5 were highly expressed during liver development (Fig. 4A). However, SFRP2 only expressed in E12.5 liver tissue, whereas the expression of SFRP3 was only detected in E12.5 and E14.5 liver tissues (Fig. 4A). Dkk1 and Dkk3 expressed in all of the isolated liver tissues. While the expression of all five SFRPs and three Dkks was readily detected in E12.5 liver, Dkk2 mostly expressed in E12.5 liver tissue (Fig. 4B). These findings strongly suggest that Wnt antagonists may play a critical role in regulating Wnt signaling activity during hepatic differentiation.

ESTABLISHMENT AND CHARACTERIZATION OF FETAL LIVER-DERIVED HPCs

Stable progenitor cell lines are essential for dissecting the role of Wnt signaling in regulating the proliferation and differentiation of HPCs. We used the previously reported reversible immortalization vector SSR #69 [Westerman and Leboulch, 1996], which employs the overexpression of SV40 large T antigen flanked by a pair of loxP sites (Fig. 5A). Using the primary fetal hepatocytes isolated from E14.5 mouse embryo fetal liver and primary hepatocytes from 2-week mouse liver tissues, we infected the cells with a retrovirus packaged SSR #69 and selected for hyrogromycin-resistant stable cell pools [Westerman and Leboulch, 1996], 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 (Fig. 5B), indicating that the integration of large T antigen is reversible. Morphologically, the immortalized stable cells retained hepatocyte-like phenotype when grown into confluence (Fig. 5C), in comparison with the mouse liver tumor line Hepa1–6.

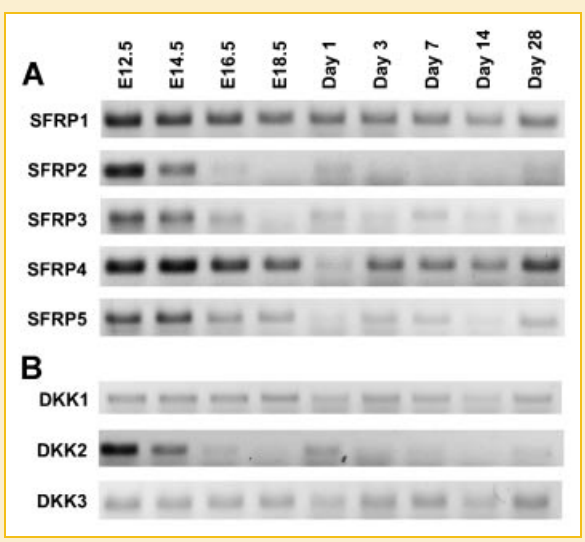

Fig. 4. Expression of Wnt antagonists during mouse liver development. The same sample set as described in Figure 1 was used for RT-PCR. A: RT-PCR analysis of the endogenous expression of the five Fzd antagonists SFRPs. B: RT-PCR analysis of the endogenous expression of Wnt co-receptor antagonists Dkks. PCR results were confirmed in three batches of independent experiments. All samples were normalized with GAPDH as shown in Figure 1.

We next examined the expression of hepatic progenitor markers and mature hepatocyte markers using immunofluorescence staining. As shown in Figure 5D, pluripotent cells marker Oct3/4 and early hepatic marker DLK were readily detected in HP14.5 cells and weakly detectable in LC14D and Hepa1–6 cells. Albumin expressed in all three lines, whereas the mature hepatocyte marker UDP-glucuronosyl transferase (UGT1A) was expressed at the highest level in Hepa1–6. Further RT-PCR analysis demonstrated that HNFs and AFP were expressed in HP14.5 cells, while the liver tumor line Hepa1–6 exhibited the highest level of expression (Fig. 5E). The early hepatic markers Thy1, CD34, and N-CAM were highly expressed in HP14.5 cells, whereas their expression was also detectable in LC14D (Fig. 5E). The hepatic marker Hhex was highly expressed in Hepa1–6 cells. Mature hepatic marker ApoB was undetectable in HP14.5 cells, and weakly expressed in Hepa1–6 cells. Furthermore, the Dex-induced expression of early and late liver markers was also analyzed and the results were consistent with the conclusion that HP14.5 cells represent the early stages of hepetoblastic progenitor cells (Supplemental Fig. 1A). The Dex-induced indocyanine green (ICG) uptake by the HP14.5 cells was further tested, and we found that there was a significant increase in ICG uptake in a time-dependent manner (Supplemental Fig. 1B), suggesting that Dex can induce HPCs into mature hepatocytes. Taken together, these results suggest that the immortalized stable HP14.5 cells retain many if not all of the hepatic progenitor phenotypes.

DISTINCT EXPRESSION PATTERNS OF WNT ANTAGONISTS IN STABLE HPCs

We next sought to determine the expression patterns of Wnt signaling components in the stable HPCs. As shown in Figure 6A, the expression of 6 of the 19 Wnt genes (e.g., Wnt1, 2, 5A, 9B, 10A, and 10B) was readily detected in HP14.5 cells. Most of the 10 Fzd receptors and LRP5/6 co-receptors were highly expressed in HP14.5 cells (Fig. 6B). Among the five SFRP antagonists, SFRP1, 2, and 4 were highly expressed, while the expression of SFRP3 and 5 was not detectable in HP14.5 cells (Fig. 6C). Dkk2 highly expressed in HP14.5 cells, whereas Dkk1 was expressed at the lowest level, and Dkk3 expression was readily detectable (Fig. 6C). Furthermore, the expression of four known Wnt targets, c-Myc, Axin2, Sox9, and Nanog was readily detected in HP14.5 cells (Fig. 6D). It is noteworthy that the expression of Wnt signaling components (especially for the antagonists) in HP14.5 cells shared an overlapping but slightly different profile from that of the freshly isolated fetal liver tissues. For example, SFRP2 and Dkk2 exhibited rather high levels of expression in HP14.5 cells while their expression was only detected in the early stage (e.g., on or before E14.5) and almost undetectable in postnatal liver tissues (Fig. 4 vs. Fig. 6D). Two possibilities may be partially accountable for the expression differences. First, the in vitro culture conditions may affect the gene expression profile of HP14.5 cells. Secondly, the freshly isolated liver tissues contained non-hepatocyte components (such as liver stellate, Kupffer cells, parenchymal cells, and sinusoidal endothelial cells) and thus the expression patterns were representation of the mixed cell types.

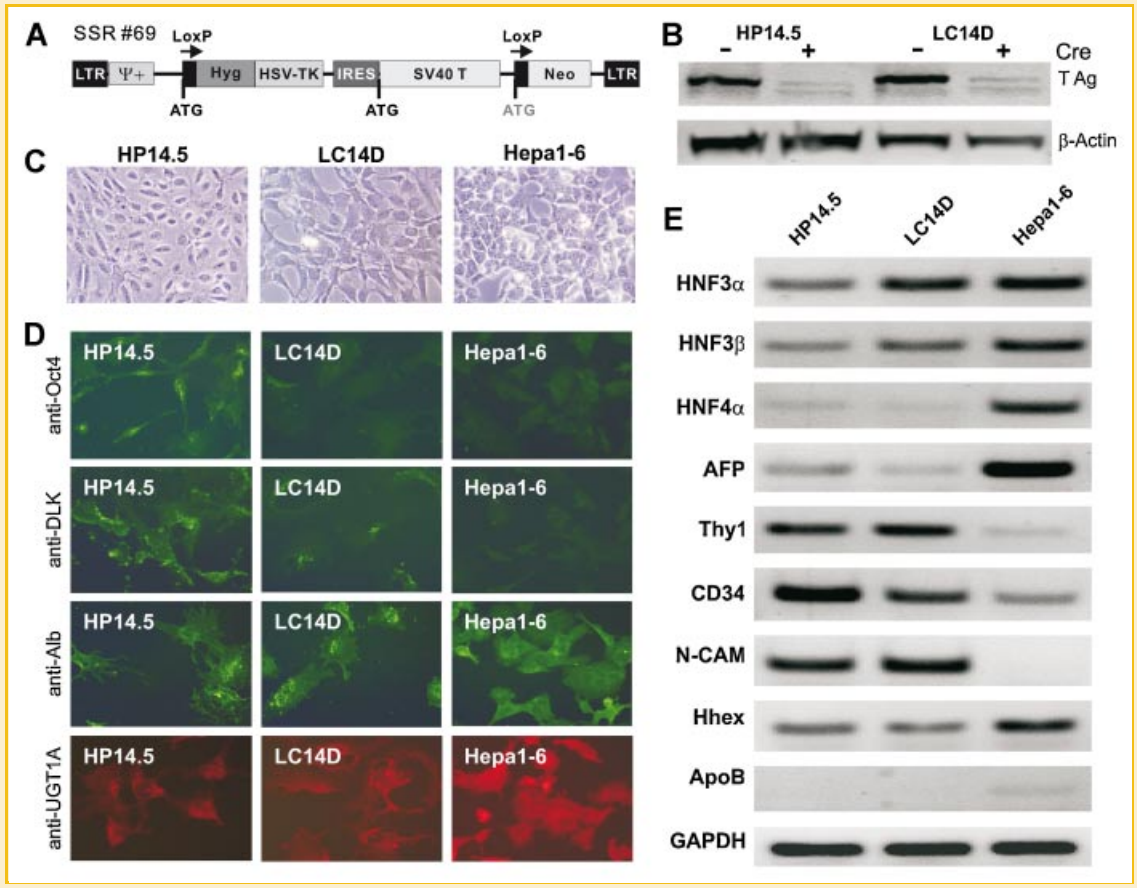

Fig. 5. Establishment and characterization of stable HPCs. A: Schematic representation of the retroviral vector SSR #69 for reversible immortalization [Westerman and Leboulch, 1996]. B: Removal of SV40 T antigen expression in the stable cells. HP14.5 and LC14D cells were infected with or without Ad-Cre for 36 h. Cells were lysed and subjected to PAGE and Western blotting using anti-large T antibody. Equal loading was confirmed by anti-β-actin Western blotting. C: Morphology of stable lines HP14.5 and LC14D. Hepa1–6 was used as a control. D: Immunofluorescence staining of stem cell markers and mature hepatocyte markers in HP14.5, LC14D, and Hepa1–6 cells. Fixed cells were probed with antibodies against Oct3/4, DLK, Alb, and UGT1A, followed by staining with FITC (for Oct4, DLK, and Alb) or Texas Red-labeled (for UGT1A) secondary antibodies. E: RT-PCR analysis of endogenous expression of HNFs and early and late markers in HP14.5, LC14D, and Hepa1–6 cells. All RT-PCR samples were normalized with GAPDH (E). RT-PCR results were confirmed in three batches of independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Nonetheless, our results indicate that HP14.5 cells possess the essential features of HPCs and exhibit a distinct expression pattern of Wnt antagonists.

Wnt ANTAGONIST SFRP3 INHIBITS HEPATIC DIFFERENTIATION

We next sought to determine the role of Wnt antagonists in hepatic differentiation. Dexamethosome (Dex) is a known inducer of hepatic differentiation [Strick-Marchand and Weiss, 2002]. When HP14.5 cells were treated with Dex (1.0 μM), the expression of AFP and UGT1A1 was induced as shown by Western blotting analysis (Fig. 7A). In order to quantitatively assess the hepatic differentiation, we constructed an Alb promoter-driven Gaussia luciferase reporter (Alb-GLuc) (Fig. 7B). HP14.5 cells were transfected with Alb-GLuc reporter plasmid and infected with Ad-GFP (−Cre) or Ad-Cre (+Cre) in the presence of Dex. Gaussia luciferase activity was assayed at days 2, 5, 7, and 9 after treatment, and the Alb promoter-driven Gaussia luciferase activity was not affected by Cre expression in HP14.5 cells (Fig. 7B) ($P < 0.2$). Overall, these results suggest that the hepatic differentiation of HP14.5 cells was not affected by the expression of SV40 large T antigen.

We next examined the effect of SFRP3 on HPCs. HP14.5 cells were transfected with Alb-GLuc reporter plasmid and infected with Ad-GFP or Ad-SFRP3 in the presence of Dex. Gaussia luciferase activity was assayed at days 4, 7, and 9. We found that the Alb-GLuc activity decreased by at least 50% in the HP14.5 cells infected with Ad-SFRP3 ($P < 0.05$–0.001) (Fig. 7C). Lastly, we tested the effect of SFRP3 on 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 infected HP14.5 cells with Ad-GFP or Ad-SFRP3 and treated with Dex. When the cells were subjected to PAS staining, positive stains were significantly decreased in Ad-SFRP3 infected HP14.5 cells (Fig. 7D). Thus, we have demonstrated that SFRP3 effectively inhibits the terminal differentiation of HPCs.

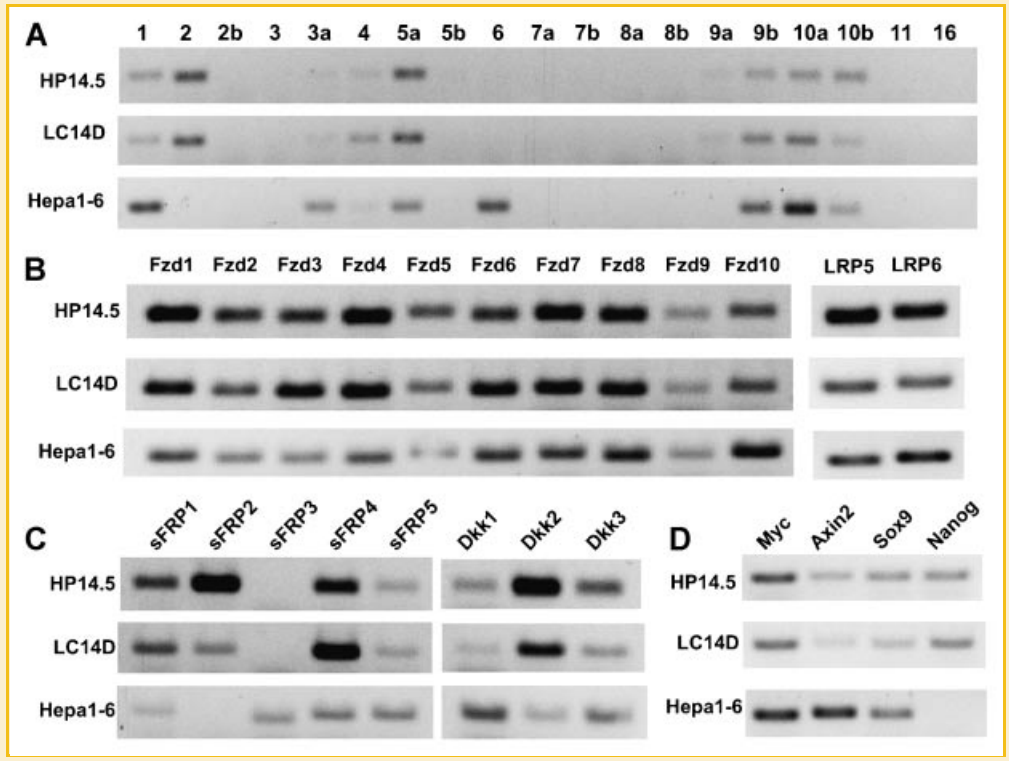

Fig. 6. Expression of Wnts, Wnt receptors, Wnt antagonists, and Wnt target genes in HPCs. A: RT-PCR analysis of the endogenous expression of 19 Wnt genes in HP14.5, LC14D, and Hepa1–6 cells. The assay conditions were the same as described in Figure 2. B: RT-PCR analysis of the endogenous expression of Wnt receptors and co-receptors in HP14.5, LC14D, and Hepa1–6 cells. The assay conditions were the same as described in Figure 3. C: RT-PCR analysis of the endogenous expression of Wnt antagonists SFRPs and DKKs in HP14.5, LC14D, and Hepa1–6 cells. The assay conditions were the same as described in Figure 4. D: Endogenous expression of known Wnt target genes in HP14.5, LC14D, and Hepa1–6 cells. All RT-PCR samples were normalized with GAPDH (Fig. 5E). PCR results were confirmed in three batches of independent experiments.

DISCUSSION

In this report, we investigated the distinct expression patterns and the functional role of Wnt antagonists in hepatic differentiation of HPCs. Using freshly isolated mouse liver tissues derived from E12.5 to PD28, we demonstrated that 13 of the 19 Wnt genes and almost all of Wnt receptors and co-receptors were expressed in most of the tested liver tissues. However, the expression of Wnt antagonists SFRP2, SFRP3, and Dkk2 was only detected in the early stages of mouse liver development. Using the established stable hepatic progenitor HP14.5 cells, we demonstrated that the stable HPCs expressed early liver stem cell markers (e.g., DLK, Oct3/4, Thy1, and N-CAM) but not late liver markers (e.g., AFP, ApoB, and UGT1A). Dex was shown to effectively induce terminal differentiation of HP14.5 cells into mature hepatocytes, as evidenced by the elevated expression of liver late markers and PAS-positive staining. However, the Dex-induced Alb expression and glycogen synthesis in HP14.5 cells were significantly inhibited by exogenous expression of SFRP3.

Our results suggest that a tight regulation of Wnt signaling activity may be critical to mouse liver development. More studies are warranted to determine whether other Wnt antagonists (e.g., SFRP2, SFRP5, and Dkk2) play any role in regulating hepatic differentiation of HPCs. Future investigations should also be directed to demonstrate if Wnt antagonists play any important role in liver development and hepatic differentiation in vivo. It would be of great interest to investigate liver and stage-specific deletions of these Wnt antagonists.

Wnt/β-catenin pathway may play an important role in embryonic liver development [Thompson and Monga, 2007; Zaret, 2008; Zaret and Grompe, 2008]. Conditional activation and deletion studies in mice have demonstrated that β-catenin plays a role in postnatal spurt in hepatic growth during the first month after birth [Cadoret et al., 2001; Tan et al., 2006, 2008]. The interaction between β-catenin and adenomatous polyposis coli (APC) may play a critical role in liver zonation process [Benhamouche et al., 2006]. Interestingly, although many Wnt genes and their receptors have been genetically deleted [Grigoryan et al., 2008], only β-catenin has been conditionally deleted or activated in mouse liver models [Cadoret et al., 2001; Harada et al., 2002; Apte et al., 2006, 2007; Tan et al., 2006, 2008; Sekine et al., 2007]. It would be of interest to examine the individual *Wnt* or *Fzd* genes for their role in normal liver development and in hepatic diseases.

Understanding the molecular mechanisms behind Wnt-regulated hepatic differentiation of HPCs may provide insights about the pathogenesis of liver disorders [Schmelzer et al., 2006]. Although the functional role of liver stem cells in liver regeneration remains controversial [Zaret and Grompe, 2008], HPCs are a promising source for liver repopulation after cell transplantation [Sell, 2001; Fausto and Campbell, 2003; Walkup and Gerber, 2006; Oertel and

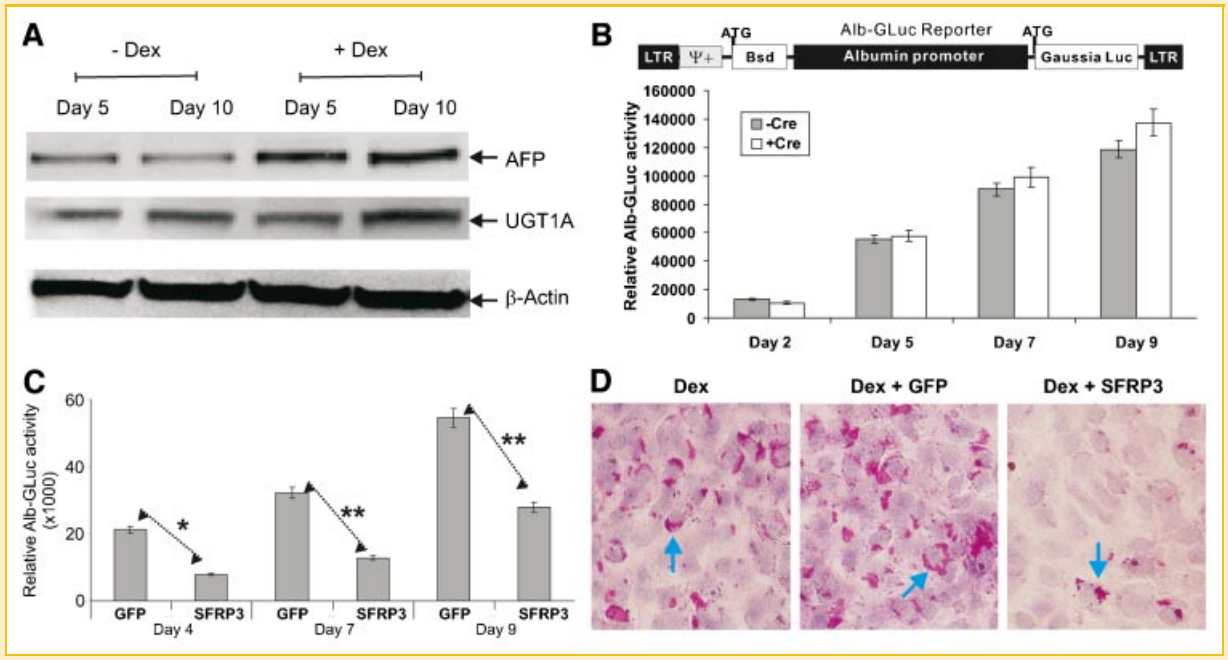

Fig. 7. Wnt antagonist SFRP3 inhibits hepatic differentiation. A: Dex-induced differentiation of HPCs. HP14.5 cells were treated with or without Dex (1 μM). Cells were collected and subjected to PAGE–Western blotting using anti-AFP, UGT1A, or β-actin antibody. B: Hepatic differentiation was not affected by SV40 large T expression. An albumin promoter-driven Gaussia luciferase reporter (Alb-GLuc) is schematically shown. HP14.5 cells were transfected with Alb-GLuc and infected with Ad-GFP (−Cre) or Ad-Cre (+Cre) in the presence of Dex (1.0 μM). Gaussia luciferase activity (mean ± SD) was assayed. Each assay condition was performed in triplicate. C: SFRP3 inhibits the differentiation of hepatic progenitor cells. HP14.5 cells were transfected with Alb-GLuc and infected with Ad-GFP (GFP) or Ad-SFRP3 (SFRP3). Gaussia luciferase activity (mean ± SD) was assayed at the indicated time points. Each assay condition was performed in triplicate. Differences in Gaussian luciferase activity between GFP and SFRP3 groups were subjected to statistical analysis. $^{*}P < 0.05$; $^{**}P < 0.001$. D: SFRP3 inhibits terminal hepatic differentiation. HP14.5 cells were infected with Ad-GFP (GFP) or Ad-SFRP3 (SFRP3) and treated with Dex (1.0 μM). At day 10, cells were fixed and subjected to PAS staining. Positive stains (purple red) are indicated by arrows. The staining results were confirmed in three batches of independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Shafritz, 2008]. The challenges are to understand the molecular mechanisms underlying hepatic differentiation and to identify factors, including components of Wnt pathways, which can effectively induce and direct HPCs to hepatocytes-lineage. Wnt/β-catenin signaling has been implicated in stellate cell activation and potentially hepatic fibrosis [Jiang et al., 2006]. Interestingly, it has been recently reported that SFRP3 was expressed in activated stellate and Kupffer cells but not in their resting counterparts in adult mouse liver [Zeng et al., 2007]. Aberrant activation of Wnt/β-catenin pathway has been implicated in hepatoblastomas, hepatocellular carcinoma, and cholangiocarcinomas [Luu et al., 2004; Clevers, 2006; Luo et al., 2007a; Thompson and Monga, 2007].

In summary, we have demonstrated that several Wnt antagonists only express in the early stage of liver development, and that exogenous expression of SFRP3 effectively inhibits hepatic differentiation of HPCs. We are characterizing individual clones for their progenitor properties. Lastly, we have not investigated the molecular mechanisms behind the decreased expression of Wnt antagonists as hepatocytes mature. Although the upstream regulatory events of Wnt antagonists can be complex, one potential cause is epigenetic regulation because methylation of SFRP promoter regions has been frequent in colon cancer [Clevers, 2006; Luo et al., 2007a]. Nonetheless, our results reaffirm the important role of Wnt/β-catenin in liver development and hepatic differentiation.

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