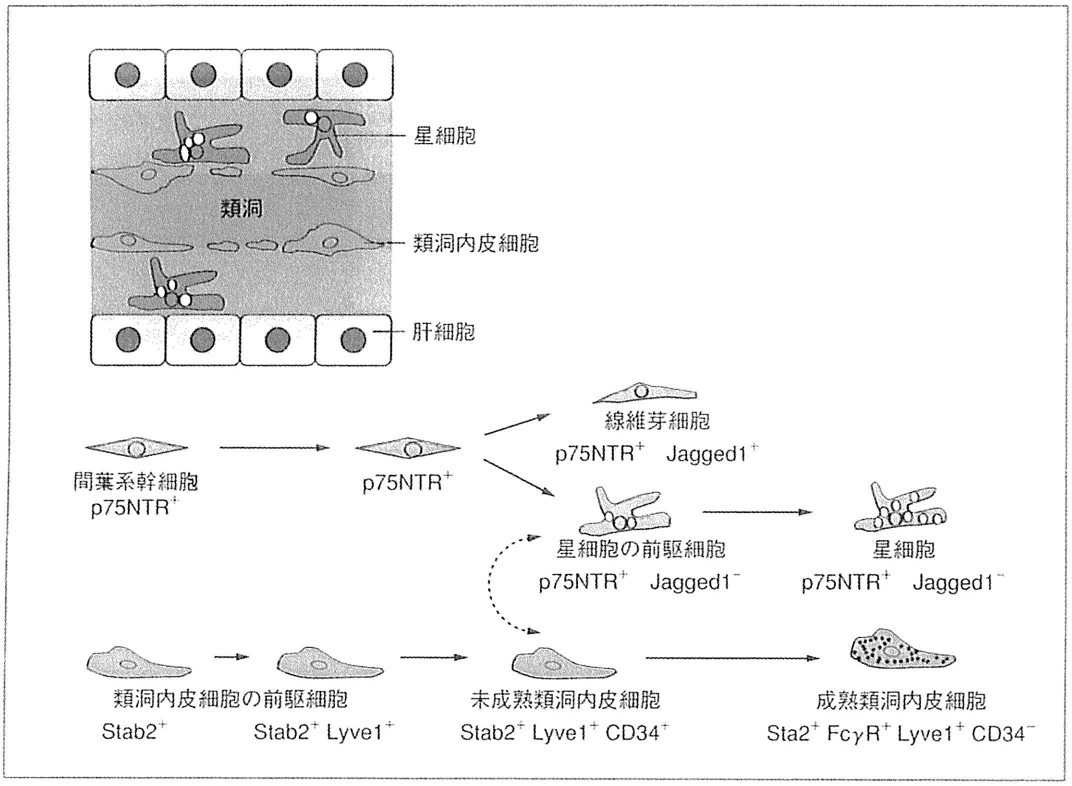
図2　類洞の構造と類洞壁細胞の分化



類洞壁を構成する類洞内皮細胞は基底膜がなく有窓構造があり，血流と肝細胞との間の物質の直接的な交換が可能である．星細胞は肝障害によりマトリックスを産生して肝線維化の中心となる細胞である．類洞内皮細胞の細胞膜抗原は分化に伴い変化する．星細胞と門脈域の線維芽細胞は共に p75NGF 陽性の間葉系細胞から派生する．類洞内皮細胞と星細胞は E10 日ではすでに接触しており，類洞形成が始まっている．

一方，成体肝臓の類洞内皮細胞は線維化，肝硬変などの病変時においては基底膜が形成され，fenestrae を有しない毛細血管へと変化することが知られている．ジメチルニトロソアミン（DMN）を投与した肝臓では，Stab2 の発現量に変化は認められないものの，CD34 および Lyve-1 の発現は顕著に亢進する．このように，肝類洞内皮細胞は発生ステージや病態により，その表現型が大きく変化する．新たに同定されたマーカーの発現を指標にすることで，発生および病変時における肝特異的内皮細胞の形質発現の変化の解析が可能となった[16]．

星細胞は類洞内皮細胞と肝細胞との間に存在する間葉系細胞であり，ビタミンA貯蔵細胞としても知られている．肝臓の線維化とともに星細胞は形質転換して線維芽細胞様の形態を呈し，コラーゲンなど

細胞外マトリックスの産生を行う．肝臓の線維化が進むと，本来基底膜を有しない類洞に基底膜様構造物が出現し，類洞の capillarization が起る．これにより，肝細胞と類洞の間に線維が蓄積し，類洞の血流と肝細胞との間の物質交換が著しく障害される．類洞の血流を左右する星細胞は，肝臓の命運を握っている重要な細胞である．しかし，この細胞の起源や類洞内皮細胞とともに類洞を形成するプロセスに関する研究は少ない．

NGF の低親和性受容体である p75NTR が星細胞に発現しており，その抗体を使ってマウス胎児から星細胞の前駆細胞を同定分離することが可能となった．E14 日の肝臓から分離した p75NTR$^+$ 細胞は vimentin や desmin などの間葉系細胞に特有の遺伝子を発現しており，そのうち 10％ 程度が油滴をためていること，さらに分離した細胞を培養すると成体肝臓の星細胞が発現する GFAP を発現することから，E14 日の p75NTR$^+$ 細胞は星細胞の前駆細胞であることが強く示唆された15)．

さらに，p75NTR の発現は E10 日ですでに認められ，E12 日では肝臓全体に分布し，E14 日では実質域と門脈域に発現する．門脈周辺の線維芽細胞は細胞外マトリックス産生を行うことで肝線維化を引き起したり，胆管の障害からの再生を促進したりすると考えられている．この間葉系細胞も p75NTR を発現しており，星細胞と起源を同じにする可能性が示唆された．さらに，この門脈域の p75NTR 陽性細胞は Jagged1 を発現しており，上記の胆管形成を誘導すると考えられる．一方，実質域の p75NTR$^+$ 細胞は E10 日ですでに類洞内皮細胞と接しており，この時期にすでに類洞形成が始まっていることが示唆された15)．

おわりに

以上，細胞膜抗原の同定をそれらに対するモノクローナル抗体を利用した細胞の同定と分離により得られた肝発生における肝臓構成細胞の性状変化を概説した．現時点ではまだ細胞種の性状の記述が中心ではあるが，今後は細胞膜抗原の発現により，肝臓の構成細胞種あるいは分化段階の異なる細胞を同定・分離する方法をさまざまな遺伝子改

変マウスの解析に取り入れることで，肝臓の発生・分化・再生の分子機構の理解が進むことが期待される．

なお，本稿は厚生労働省『次世代医療機器評価指標作製事業の再生医療』の報告書としてまとめたものを改編したものである．

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Liver Development and Differentiation

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Y-Box Binding Protein-1 Down-Regulates Expression of Carbamoyl Phosphate Synthetase-I by Suppressing CCAAT Enhancer-Binding Protein-Alpha Function in Mice

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BACKGROUND & AIMS: Carbamoyl phosphate synthetase-I (CPS1) is a key enzyme in the urea cycle and patients with defects in the function or expression of CPS1 suffer from hyperammonemia. CPS1 is expressed in the liver at neonatal and adult stages in a CCAAT enhancer-binding protein-alpha (C/EBPα)-dependent manner. Despite expression of C/EBPα, CPS1 is not expressed in fetal liver, indicating an additional factor is involved in the regulation of CPS1 expression. The aim of this study was to elucidate the mechanism of CPS1 expression. **METHODS:** Microarray was performed to find Y-box binding protein-1 (YB-1) that was expressed in mouse fetal liver. The role of YB-1 in CPS1 expression was investigated by overexpression of YB-1 in mouse fetal liver culture and luciferase reporter assays using the CPS1 promoter. Chromatin immunoprecipitation assay was used to examine recruitment of YB-1 to the CPS1 promoter in vivo. **RESULTS:** Expression of YB-1 and CPS1 was inversely correlated in vivo, and YB-1 inhibited CPS1 expression and ammonia clearance in fetal liver culture. Although YB-1 was not expressed in adult liver, acute liver injury up-regulated YB-1 and down-regulated CPS1, accompanying an increase of the serum ammonia level. YB-1 inhibited C/EBPα-induced transcription from the CPS1 promoter via the Y-box near the C/EBPα-binding site. Chromatin immunoprecipitation assays demonstrated that YB-1 was recruited to the CPS1 promoter in fetal and injured adult liver, but not in normal adult liver. **CONCLUSIONS: YB-1 is a key regulator of ammonia detoxification by negatively regulating CPS1 expression via suppression of C/EBPα function.**

The liver functions as a major hematopoietic organ in the fetus and acquires various metabolic functions during perinatal and neonatal stages. Among numerous liver functions in adult, ammonia detoxification is essential for life and excess ammonia in the body can be fatal. The nervous system is particularly sensitive to excessive ammonia and impaired clearance of ammonia is a major cause of hepatic encephalopathy.[1,2] A majority of the excess ammonia in mammals is converted into urea via the urea cycle, also known as the ornithine cycle, in the liver. Urea is then excreted by the kidneys. Glutamine synthetase (GS) also contributes to the metabolism of ammonia by converting it to glutamine. The urea cycle includes 5 major enzymes: carbamoylphosphate synthetase-I (CPS1), ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinate lyase, and arginase. CPS1 catalyzes the first and rate-limiting step of the urea cycle, the formation of carbamoyl phosphate from ammonia and bicarbonate. It has been well established that defects in the function or expression of CPS1 causes hyperammonemia, which can result in brain damage and even death.[2,3] CPS1-deficient mice die soon after birth with overwhelming hyperammonemia.[4] These observations indicate that regulation of CPS1 expression is crucial for ammonia detoxification.

Analysis of the CPS1 promoter using hepatoma cell lines revealed that there are several *cis* regulatory elements required for the maximum promoter activity: a GAGA box at around −55 bp, a CCAAT enhancer-binding protein-alpha (C/EBPα) protein binding sequence at around −110 bp, and a glucocorticoid-response element (GRE) at around −95 bp. In addition to the promoter region, there is an enhancer region at −6.3kb from the transcription starting site, which contains C/EBP protein binding sites, GRE, FoxA/HNF3 binding sites, and cyclic AMP-response elements (see Figure 3*A*).[5–7] The −6.3 kb-distal enhancer region was suggested to interact with the proximal promoter by a glucocorticoid receptor (GR) homodimer that binds both regions via GREs and to induce CPS1 transcription.[8] C/EBPα-deficient mice lack CPS1 expression and exhibit hyperammonemia,[9] indicating that C/EBPα is essential for CPS1 expression. During liver development, CPS1 is expressed at the neonatal

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Abbreviations used in this paper: CPS1, carbamoyl phosphate synthetase-I; C/EBPα, CCAAT enhancer-binding protein-alpha; CCl_4, carbon tetrachloride; ChIP, chromatin immunoprecipitation; Dlk, Delta-like protein; EGFP, enhanced green fluorescent protein; G6Pase, glucose-6-phosphatase; GS, glutamine synthetase; GR, glucocorticoid receptor; GRE, glucocorticoid-response element; GS, glutamine synthetase; HCC, human hepatocellular carcinoma; MDR1, multidrug resistant 1; PEPCK, phosphoenolpyruvate carboxykinase; TAT, tyrosine aminotransferase; YB-1, Y-box binding protein-1.

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stage; C/EBPα as well as other positive regulators—GR, HNF3—are expressed in the fetal liver. Thus, an additional factor is suggested to be involved in the regulation of CPS1 expression.

By microarray analysis of mouse hepatocytes at different developmental stages, we found that Y-box binding protein-1 (YB-1) was highly expressed in fetal liver and was down-regulated along with development. Because YB-1 expression was inversely correlated with CPS1 expression during development, in this study, we examined the role of YB-1 in CPS1 expression. YB-1 is a multifunctional DNA/RNA binding protein belonging to the cold shock domain family, which is widely distributed from bacteria to mammals.[10] YB-1 is a transcription factor recognizing the *cis*-regulatory element, Y-box, defined as a reverse CCAAT box or ATTGG.[11] YB-1 is also an RNA-binding protein involved in the splicing, transport, and translation of mRNA and is implicated in DNA repair.[12,13] Y-box is present in the promoter of many genes such as the multidrug-resistant 1 (*MDR1*), major histocompatibility complex class II, epidermal growth factor receptor, DNA polymerase, thymidine kinase, and topoisomerase II genes. YB-1 promotes proliferation of tumor cells, such as breast, ovarian, lung, and prostate cancers, and up-regulates the expression of *MDR1*.[14] YB-1 is also considered as a tumor marker of human hepatocellular carcinoma (HCC).[15] In an inflamed liver, it suppresses the formation of collagen and modulates fibrosis.[16] The liver in YB-1-deficient mice is small and the mutant mice die soon after birth.[17] These results suggest that YB-1 plays an important role in the liver. However, its function during liver development and in adults remains elusive. Because YB-1 expression was inversely correlated with CPS1 expression, we investigated role of YB-1 in CPS1 expression and revealed that YB-1 represses CPS1 expression and plays a critical role in the metabolism of ammonia in developing hepatocytes as well as acute hyperammonemia in mouse adult liver.

Materials and Methods

Mice, Cells, and Cell Culture

C57BL/6 mice (Nihon SLC, Hamamatsu, Japan) were used for all the experiments under Guidelines of the University of Tokyo for Animal Care. 293T and HepG2 cells and BOSC23 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum and 50 μg/ml of gentamycin. Mouse fetal liver cells were cultured as reported.[18] Hepatic progenitor cells proliferating on laminin cells were cultured as reported.[19]

Microarray Analysis

Mouse hepatoblasts were isolated from E12.5 and E17.5 fetal liver using anti-mouse Delta-like protein (Dlk) monoclonal antibody according to a previous report[19] and dissolved in Trizol reagent. The cDNA samples synthesized from total RNA were used for a microarray analysis with the mouse GEM2 microarray. Microarray data can be assessed by the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=fdilvegmiwowqvy&acc=GSE13363.

Luciferase Reporter Assay

Transfection was performed using Lipofectamine plus reagent, according to the manufacturer's instructions (Invitrogen). The luciferase assay was performed according to the manufacturer's instructions (Promega, Madison, WI). Each experiment was performed in triplicate. All experiments using 293T and HepG2 cells were performed with at least three different cell preparations.

Plasmids and Antibodies

Mouse C/EBPα cDNA was cloned by polymerase chain reaction into a pcDNA3 vector (Invitrogen) based on the reported sequence.[20,21] YB-1 and GR cDNAs were inserted into pcDNA3 (Invitrogen). A retroviral vector for YB-1 expression was constructed using the plasmid pMXIG. The proximal region (−600 to +10) and distal-proximal region (−6.3 kb element; 451 bps)[6] were inserted into pGL4-luc vector (Promega). The distal element (−6.3 kb element; 451 bps) was connected to the proximal region as shown in Figure 3C and cloned into pGL4-luc (Figure 3*B*, *C*). Antibodies used in this study were: anti-C/EBPα (14AA; Santa Cruz Biotech, Santa Cruz, CS), anti-YB1 (ab12148; Abcam, Cambridge, MA), anti-CPS1 (E-20, Santa Cruz Biotech), and anti-actin (I-19, Santa Cruz Biotech).

Northern and Western Blotting

As described previously,[21] Northern and Western blottings were performed. Total RNA from E14.5, E17.5, neonatal, and adult mouse livers (4 and 8 weeks old), and also from cultured cells was extracted by using Trizol solution (Invitrogen) and then analyzed by Northern blotting. Livers from E14.5, E17.5, neonatal, and adult mice (4 and 8 weeks old) were homogenized and lysed. Protein samples were subjected to sodium dodecyl sulfate-PAGE, and immunoblotted using antibodies against YB-1, CPS1, or C/EBPα.

Retroviral Infection

Retroviral vectors were transfected into BOSC23 cells by lipofection and the viral particles were harvested after 2 days. The particles were collected by centrifugation at 6000 *g* for 16 hours at 4°C and pellets were resuspended in fetal hepatocyte culture medium. Fetal hepatocytes grown in 6-well plates for 2 days after plating were incubated with the viral suspension. After 2 days, the culture medium was changed to a virus-free fetal hepatocyte culture medium.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay was performed with a kit (Upstate), and also described previously.[21] Polymerase chain reaction was performed with the following primers:

CPS1: sense (-300), 5′-TGCATTATTAGCAAGGTACTGCCC-3′; anti-sense (-50), 5′-TTCCTTAGCCCCTCCTCCCAAGCTG-3′.

β-Actin: sense (-75), 5′-GTTCCGAAAGTTGCCTTTTATG-3′; anti-sense (+252), 5′-ATGTGGCTGCAAAGAGTCTACA-3′.

Mouse Liver Injury by CCl_4 and Acetaminophen

Liver injury was induced by an IP injection at a dose of 500 mg/kg body weight acetaminophen dissolved in phosphate-buffered saline (Sigma, St Louis, MO) or a 20% (v/v) solution of CCl_4 (Wako Pure Chemicals, Osaka, Japan) in corn oil at a dose of 7 μL/g body weight.[22]

Ammonia and Urea Levels

To examine the cellular activity to clear ammonia and produce urea, fetal hepatocytes were loaded with 2 mmol/L NH_4Cl at day 7 after plating and further incubated for 0–72 hours.[23] Concentrations of ammonia in culture media were measured by the modified indophenol method using a commercial kit (Ammonia-Test Wako; Wako Pure Chemical Industries). Concentrations of urea were measured by a urea assay kit (Quantichom Urea assay kit; BioAssay systems, Hayward, CA). The blood of CCl_4-treated mice was drawn and analyzed with the same kit.

Results

Gene Expression Profiles of Mouse Hepatoblasts/Immature Hepatocytes During Development

Mouse hepatoblasts emerging at E8.5 from the foregut endoderm proliferate vigorously and differentiate to hepatocytes and biliary epithelial cells.[24] To find genes important for hepatocyte differentiation during development, we compared gene expression profiles of mouse hepatoblasts/immature hepatocytes at E12.5 and E17.5. Because Dlk, also known as Pref-1, is expressed in hepatoblasts/immature hepatocytes,[19] we performed a microarray analysis of the Dlk^+ cells isolated from livers at E12.5 and E17.5. Consistent with the high proliferative potential of hepatoblasts in the early stages, cyclins D1, D2, and E were highly expressed in E12.5 hepatoblasts (Table 1). Among the top 30 genes preferentially expressed in E12.5 hepatoblasts, there were several transcription factors such as HMGA1, CITED, FoxM1, GATA6, and YB-1. In this study, we have focused on YB-1.

Northern blot analysis revealed that YB-1 was highly expressed in E14.5 liver, but its expression was significantly down-regulated at E17.5 and was absent in adult liver. Consistent with a report that cyclin A expression was regulated by YB-1,[25] cyclin A was also highly ex-

Table 1. Genes Highly Expressed in E12.5 Hepatoblasts

No.	Gene name	Accession no.	No.	Gene name	Accession no.
1	Midkine	AI325764	19	Y box protein 1	AA450785
2	High mobility group AT-hook 1	AA067083	20	Serum response factor	AA415341
3	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	AA709508	21	Teratocarcinoma expressed, serine rich	AA116831
4	Cyclin D1	AA117547	22	Small inducible cytokine B subfamily (Cys-X-Cys), member 10	AI158236
5	Cyclin D2	AA879568	23	Cyclin F	AI326604
6	Cyclin E	AA437868	24	Stromal cell derived factor 1	AA066069
7	Dickkopf homolog 3	AA073904	25	Epidermal growth factor-containing fibulin-like extracellular matrix protein 2	AW210333
8	Nik related kinase	AI225895	26	Ras-GTPase-activating protein (GAP) SH3-domain-binding protein 2	AA617613
9	TEA domain family member 2	AA617316	27	Transforming, acidic coiled-coil containing protein 3	AA190123
10	Forkhead box M1	AW519587	28	EphA4	AI325333
11	Tcra enhancer-binding factor interacting protein 1	AA437646	29	Enhancer of zeste homolog 2	AA445767
12	Transmembrane 4 superfamily member 6	AA437557	30	NPC derived proline rich protein 1	AA791966
13	Cysteine rich protein 61	AA466852			
14	Integral membrane protein 2	AA387218			
15	Cyclin-dependent kinase 4	AI892404			
16	GATA-6	AA536899			
17	Pleiotrophin	AI047160			
18	CDk2-associated protein 1	AA638778			

Dlk+ hepatoblasts were isolated from E12.5 and E17.5 fetal liver using AutoMACS. Gene expression analysis was performed using a Mouse GEM2 microarray.

pressed in E14.5 liver, but was diminished at E17.5. In contrast, C/EBPα was constitutively expressed from E14.5 to adulthood, and the expression of CPS1 and GS was up-regulated at the neonatal stage (Figure 1*A*). Western blotting also confirmed that YB-1 protein was present in E14.5 and E17.5 liver, but not in neonatal liver, and 2 C/EBPα isoforms were expressed constitutively (Figure 1*B*). CPS1 was expressed from neonatal liver (Figure 1*B*). Although YB-1 mRNA expression was significantly down-regulated at E17.5, YB-1 protein was still detected at this stage, suggesting that YB-1 protein is stable in mouse hepatoblasts.

YB-1 Inhibits Expression of CPS1

To reveal the role of YB-1 in the maturation of hepatocytes, we took advantage of a primary culture system, in which fetal hepatocytes can be induced to differentiate by oncostatin M.[18] In this culture system, many metabolic enzymes such as CPS1, tyrosine aminotransferase (TAT), and glucose-6-phosphatase (G6Pase) were undetectable for the first 3 days and were detected at day 7 after plating (Figure 1*C* and *D*). By contrast, YB-1 mRNA and protein were present for the first 3 days and diminished at 7 days after plating, whereas C/EBPα was constitutively expressed during the culture period (Figure

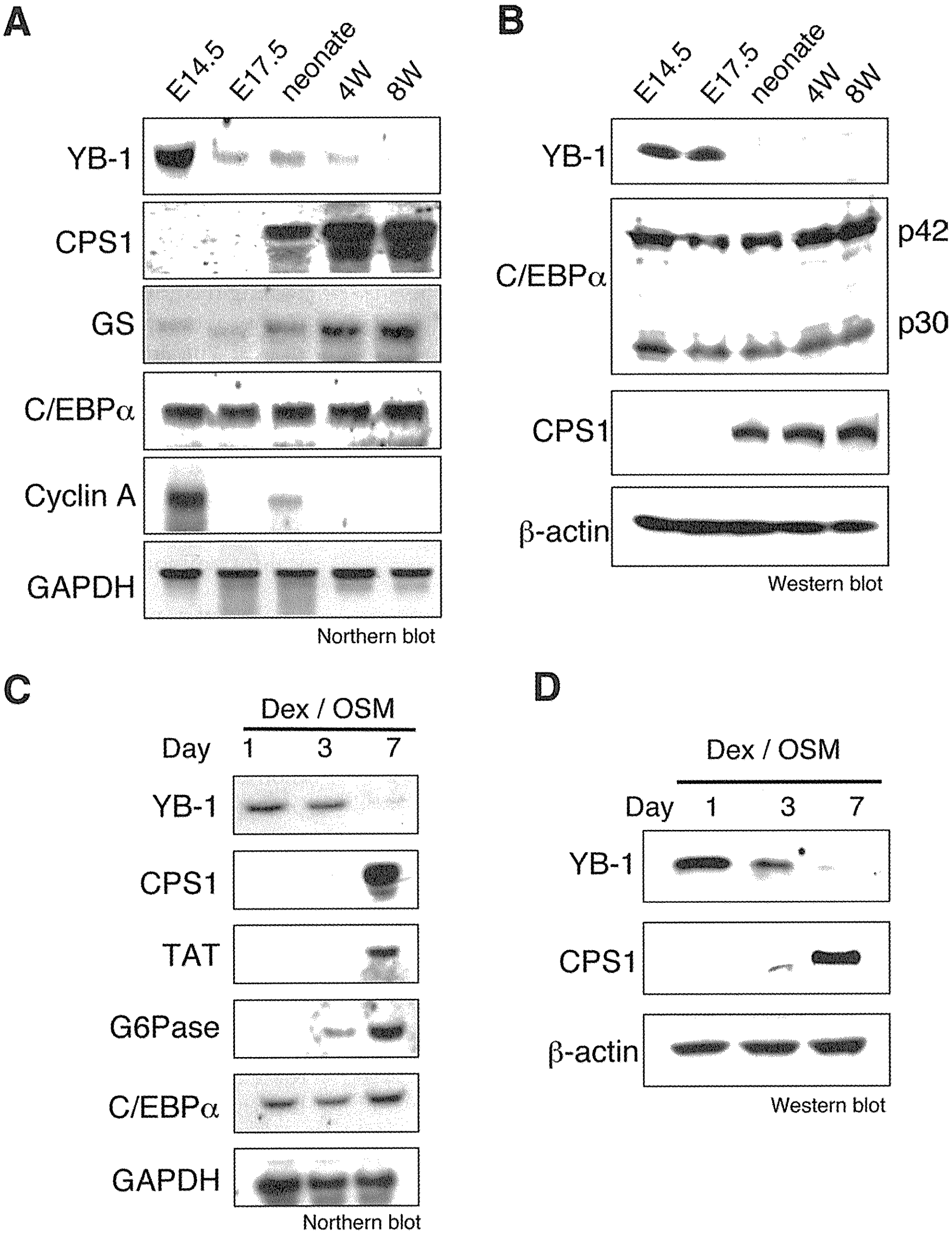

Figure 1. Expression of YB-1 during mouse fetal liver development. (*A*) Expression of various mRNA as shown during liver development by Northern blot analysis. RNA was prepared from mouse liver tissues at E14.5, E17.5, the neonatal period, and 4- and 8-week-old mice. (*B*) Protein levels of YB-1, C/EBPα isoforms (p42 and p30), CPS1, and β-actin during liver development. The protein levels in whole cell extracts from the liver at different stages were examined by Western blot analysis. (*C*) Expression of mRNA in fetal hepatic cultures. E14.5 fetal liver cells were cultured and RNA was prepared for Northern blot. (*D*) Protein levels of YB-1, CPS1, and β-actin in fetal hepatic cultures. Mouse E14.5 fetal liver cells were cultured and protein was prepared for Western blot analysis.

1C and D). The results are consistent with YB-1 expression during liver development in vivo (Figure 1A); that is, YB-1 expression is strong in immature liver, but is weak in adult liver.

To investigate the role of YB-1 protein in the maturation of hepatocytes, we overexpressed YB-1 in cultured mouse fetal liver cells using a retroviral vector encoding both YB-1 and enhanced green fluorescent protein (EGFP) proteins. The efficiency of the infection was estimated to be about 65% based on an analysis of EGFP expression by flow cytometry. In the control culture, fetal hepatocytes differentiated, as shown by the morphology of mature hepatocytes and expression of liver enzymes such as TAT, G6Pase, and CPS1. In the cultures infected with the YB-1–encoded retrovirus, the expression of a YB-1 target gene, cyclin A, was up-regulated (Figure 2A). Although the morphology of the hepatocytes (data not shown) and expression of TAT and G6Pase were similar to those in the control culture (Figure 2A), CPS1 expression was severely inhibited. In contrast with CPS1, a key enzyme for the urea cycle, expression of GS, which also contributes to ammonia clearance to a limited extent, was not affected by YB-1. In addition, YB-1 did not change the gene expression of other enzymes in the urea cycle such as ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinate lyase, or arginase (data not shown). Besides, knockdown of YB-1 expression by small interfering RNA up-regulated CPS1 expression in a hepatoblast cell line (Supplementary Figure 1). These results indicated that expression of CPS1 and YB-1 was inversely correlated in vitro as well as in vivo (Figure 1), suggesting that YB-1 is a negative regulator of CPS1 expression. By contrast, the expression of cyclin A was up-regulated by YB-1 (Figure 2A).

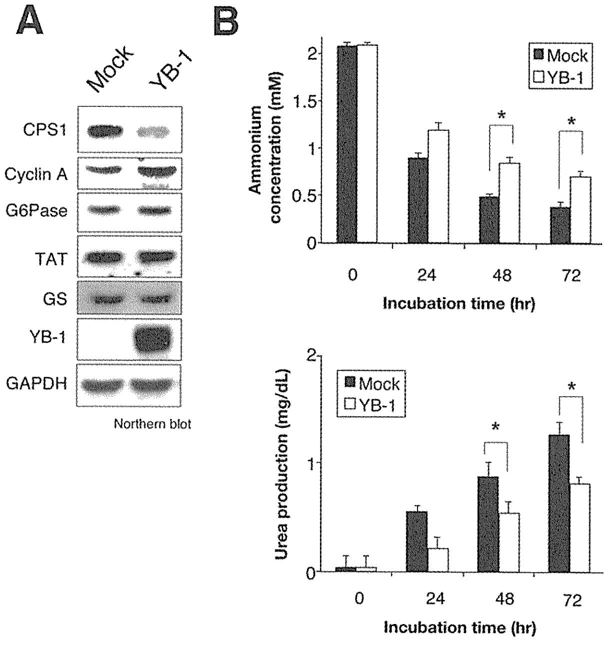

Figure 2. The effect of YB-1 on CPS1 expression and ammonia clearance in mouse fetal liver cells. (A) YB-1 was expressed using a retroviral system in the fetal liver culture. The levels of mRNA of CPS1, cyclin A, G6Pase, TAT, GS, YB-1, and GAPDH at 7 days after the infection were examined by Northern blot analysis. (B) The ammonia and urea production levels in the culture medium of fetal liver culture. The medium was changed to the one containing ammonia 3 days after the retroviral infection and the ammonia and urea levels were determined at different time points as indicated. Because serum in the culture medium contained urea, the data shown are urea produced during culture, that is, the serum urea level was subtracted from the amount of urea measured in the cultured media. The ability of the cells overexpressing YB-1 to metabolize ammonia was less than that of control cells. Error bars represent + standard deviations. *$P < .05$.

Inverse Correlation Between YB-1 Expression and Ammonia Clearance

CPS1 is a key enzyme involved in ammonia detoxification in mammals. To investigate whether YB-1 regulates ammonia levels, we evaluated the ability of hepatocytes to metabolize ammonia in the primary culture of E14.5 liver cells infected with a retrovirus encoding YB-1. After 7 days of culture, the medium was replaced with ammonia-containing medium and the ammonia concentration was measured. Ammonia-containing medium did not affect viability, gene expression, or morphology of the cultured cells (Supplementary Figure 2). The ammonia levels were higher in the culture medium infected with YB-1–encoded virus than the control culture (Figure 2B), consistent with the CPS1 expression. In contrast with ammonia levels, urea production in the culture infected with virus carrying YB-1 cDNA was lower than the control culture (Figure 2B). These results indicate that CPS1 expression modulated by YB-1 is critical for ammonia clearance and urea production.

YB-1 Inhibits C/EBPα-Mediated CPS1 Transcription

To further investigate the role of YB-1 in CPS1 expression, a luciferase reporter assay was used to determine whether or not YB-1 affected transcription from the mouse CPS1 promoter. Previous studies on the CPS1 promoter identified 2 regions important for CPS1 expression, namely, the distal −6.3-kb enhancer and the proximal promoter (Figure 3A).[6,26] First, we cloned the promoter region of the CPS1 gene covering 600 nucleotides from the transcription starting site, and inserted it into the pGL4 luciferase reporter vector, pGL4-CPS1 (proximal)-Luc (Figure 3B, *upper scheme*). Because CPS1 expression requires C/EBPα and there is a C/EBPα recognition site in the promoter fragment,[26] we examined the effect of C/EBPα on CPS1 transcription. Although luciferase was not expressed by transfection of the reporter construct with the CPS1 promoter alone, co-transfection of the C/EBPα expression vector dramatically induced the expression of luciferase from the CPS1 promoter in 293T or HepG2 cells (Figure 3B; Supplementary

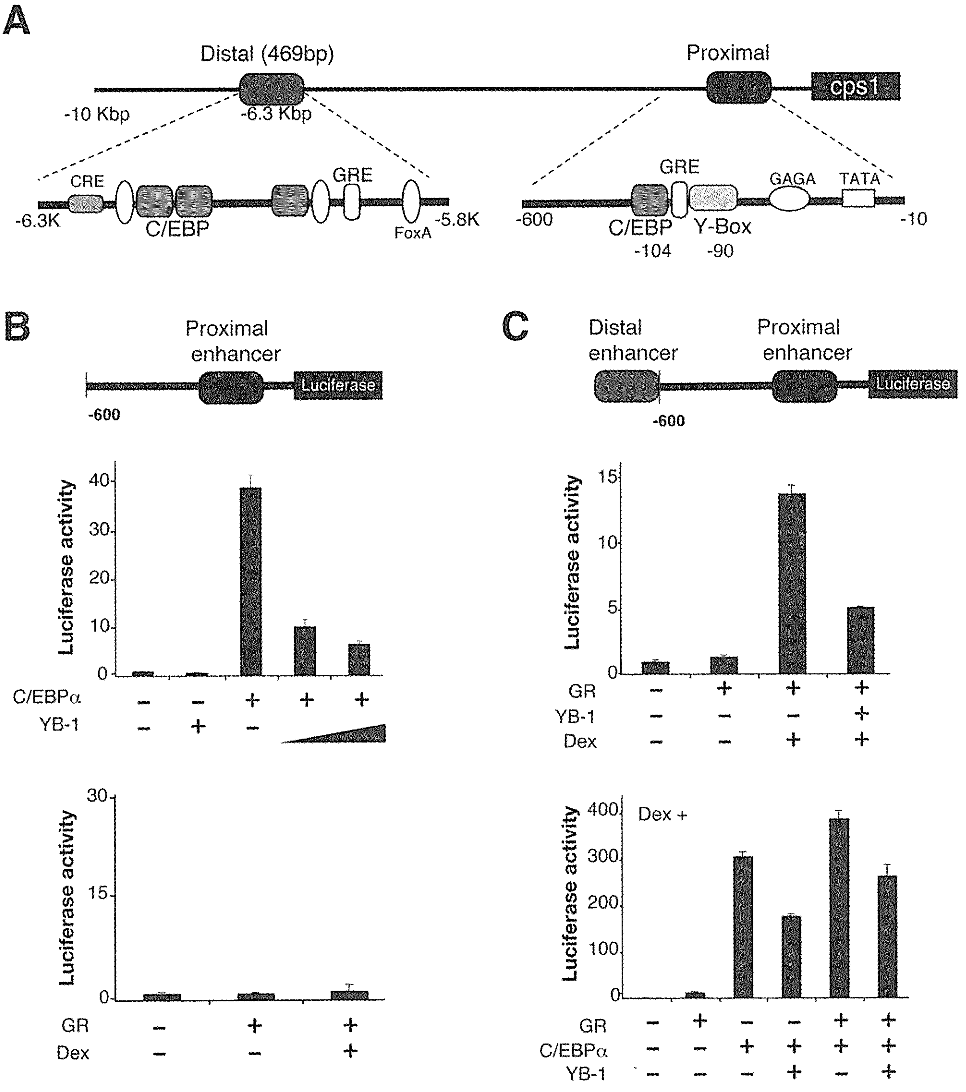

Figure 3. Suppression of transcription from the CPS1 promoter by YB-1. (*A*) The distal enhancer and proximal enhancer of the CPS1 gene promoter. (*B*) Structure of pGL4-CPS1 (proximal)-Luc vector. (*C*) Structure of pGL4-CPS1 (distal–proximal)-Luc vector. 293T cells were transfected with the CPS1 promoter-Luc vectors together with the expression vectors for C/EBPα, GR, and YB-1 as indicated. Cells were harvested for luciferase assays 48 hours after transfection. Data shown are normalized by *Renilla* luciferase activity and are mean values + standard deviations. A representative experiment performed in triplicate is shown.

Figure 3*A*). We then co-expressed YB-1 and found that C/EBPα-induced expression of luciferase was severely inhibited by YB-1 in a dose-dependent manner (Figure 3*B*; Supplementary Figure 3*A*).

It was reported that the CPS1 promoter was activated by the distal −6.3-kb enhancer (distal). The enhancer was suggested to interact with the promoter by a GR–GR homodimer that binds the GRE sites in both regions (Figure 3*A*).[8] To investigate whether YB-1 also affects the −6.3-kb (distal) enhancer, we constructed pGL4-CPS1 (distal-proximal)-Luc, which contained both distal and proximal regions (Figure 3*C*).[8] GR is an essential factor for CPS1 expression and it, in fact, induced the transcription form pGL4-CPS1 (distal-proximal)-Luc in the presence of Dex. However, GR failed to do so from pGL4-CPS1 (proximal)-Luc (Figure 3*B*). C/EBPα induced transcription from pGL4-CPS1 (distal-proximal)-Luc stronger than GR (Figure 3*C*) and co-expression of GR and C/EBPα synergistically enhanced the transcription. Furthermore, YB-1 suppressed the transcription by either GR or C/EBPα alone and also by co-expression of GR and C/EBPα (Figure 3*C*; Supplementary Figure 3*A*). These results confirmed the previous results that the distal and proximal regions cooperatively induced CPS1 expression and indicated that YB-1 is a negative regulator of CPS1 expression.

YB-1 is known to bind the Y-box sequence, namely, inverted CCAAT, to regulate gene expression.[11] We found one inverted CCAAT sequence, ATTGG, at −90 in the CPS1 promoter (Figure 4*A*), which was only 10 nucleotides from the C/EBPα recognition site, GTTGCAATTTGTAT (Figure 4*A*).[26] Therefore, we considered the possibility that YB-1 binds to this site and inhibits the transcriptional activation of CPS1 by C/EBPα. We examined whether this Y-box is responsible for the suppression of C/EBPα-induced CPS1 transcription by mutating

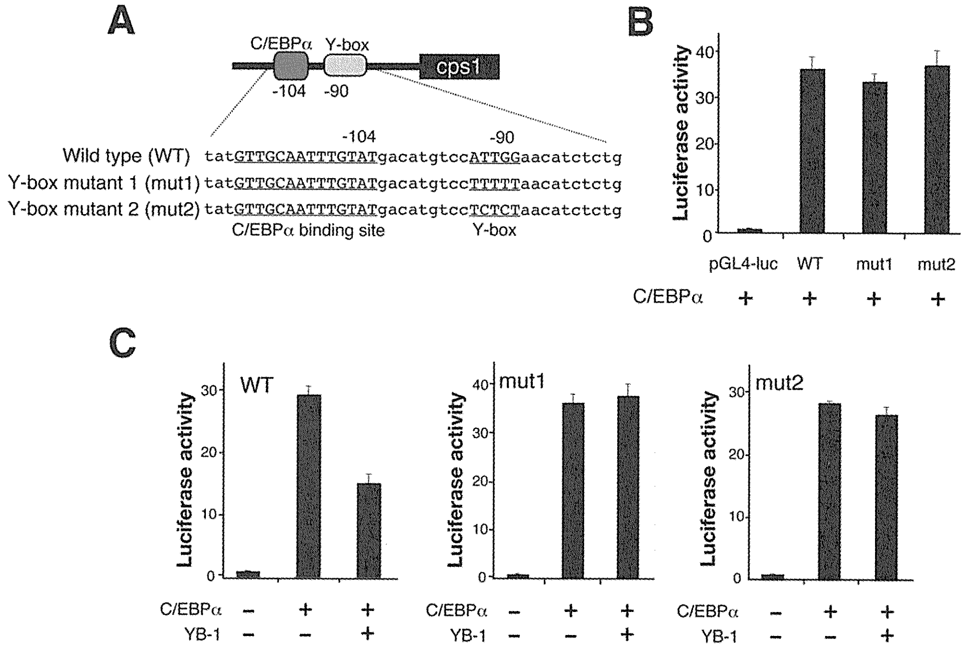

Figure 4. Suppression of transcription from the CPS1 promoter via Y-box site. (*A*) Nucleotide sequence of the promoter region of the CPS1 gene. There is an inverted CCAAT sequence at position −90 from the transcription start site of the CPS1 promoter and the C/EBPα binding site is between −104 and −117. The Y-box was mutated to TTTTT (Y-box mutant 1) and TCTCT (Y-box mutant 2). (*B*) C/EBPα and pGL4-CPS1 (proximal)-Luc plasmids were co-transfected into 293T cells with either CPS1 (proximal)-Luc (WT), Y-box mutant 1-Luc (mut1), or Y-box mutant 2-Luc (mut2) and luciferase activity was measured 48 hours later. (*C*) Wild-type, mut1, or mut2 was transfected with C/EBPα and YB-1 expression vectors as indicated and luciferase activity was measured 48 hours later. Data shown are normalized by *Renilla* luciferase activity and are mean values + standard deviations. A representative experiment performed in triplicate is shown.

the inverted CCAAT to TTTTT or TCTCT. These mutant promoters were still capable of expressing luciferase in a C/EBPα-dependent manner (Figure 4*B*); however, YB-1 failed to repress C/EBPα-dependent transcription from these 2 mutated promoters (Figure 4*D*). Roles of C/EBPα (−104) and YB-1 (−90) recognition sites in CPS1 gene enhancer were also tested by mutation analysis. Disruption of the C/EBPα site reduced the promoter activity, whereas the Y-box mutation rather enhanced the promoter activity in HepG2 cells (Supplementary Figure 3*B*). These results clearly indicated that the suppression of CPS1 promoter activity by YB-1 is mediated by the Y-box in the promoter.

YB-1 Is Recruited to the CPS1 Promoter in Fetal Liver

We examined if the YB-1 protein binds to the CPS1 promoter in E14.5 liver in vivo using ChIP assays. From E14.5 liver samples, anti–YB-1 antibody immunoprecipitated the target fragment, whereas it failed to do so in normal adult liver (Figure 5). By contrast, anti-C/EBPα antibody immunoprecipitated the target fragment from E14.5 as well as adult liver samples (Figure 5). These results indicate that YB-1 binds in vivo to the CPS1 promoter in fetal but not in normal adult liver.

YB-1 in Acute Hepatitis

Detoxification of ammonia is an essential liver function.[27] Because hyperammonemia is associated with liver injury, we examined the expression of CPS1 and YB-1 during acute liver injury induced by the administration of carbon tetrachloride (CCl_4). The serum ammonia level began to increase 24 hours after the administration of CCl_4, reached a peak at 48 hours, and then gradually declined to the basal level (Figure 6*B*). In contrast, the serum urea level was decreased at 48 hours after CCl_4 injection (Figure 6*B*). Northern blot analysis showed that the CPS1 mRNA level was transiently, but significantly, decreased in the liver at 24 hours after the CCl_4 injection. By contrast, the YB-1 and cyclin A mRNA levels were increased after the injection (Figure 6*A*, *left panel*). Protein levels of YB-1 and CPS1 were also inversely correlated (Figure 6*A*, *right panel*). The expression of C/EBPα was not changed significantly during this stage (Figure 6*A*). Moreover, ChIP assays showed that anti–YB-1 antibody precipitated the CPS1 promoter fragment in injured liver, but not in normal adult liver (Figure 6*C*). These results together indicate that YB-1 is recruited to the CPS1 promoter and regulates the metabolism of ammonia in injured liver.

As another model of liver injury, we employed acetaminophen-induced acute liver injury, in which CPS1

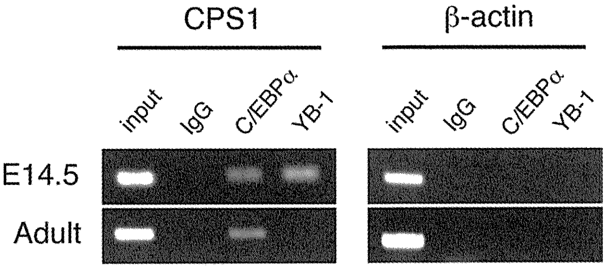

Figure 5. Recruitment of YB-1 in liver. Recruitment of C/EBPα and YB-1 to the target DNA demonstrated by chromatin immunoprecipitation assays. The association of C/EBPα and YB-1 with the endogenous CPS1 promoter (−300∼−50) was detected by chromatin immunoprecipitation assays as described in Materials and Methods. The β-actin promoter fragment (−75∼+252) failed to recruit C/EBPα and YB-1, indicating specific recruitment of these proteins to the CPS1 promoter.

activity is inhibited and concomitantly acute hyperammonemia is induced.[28] We found that the administration of acetaminophen up-regulated YB-1 expression and down-regulated CPS1 expression (Figure 6*D*). These results indicate that YB-1 expression is inversely correlated with CPS1 expression in adult mouse liver.

Discussion

YB-1 contains a cold shock domain that binds DNA and has been shown to regulate the expression of many genes, including the cyclin A, cyclin B, and *MDR1* genes.[25,29] YB-1 is highly expressed in fetal liver, injured liver (Figures 1*A* and *B*; and 6*A* and *D*), and also in HCC[15]; however, the role of YB-1 in the liver remained unclear. This study reveals a previously unrecognized important function of YB-1 for ammonia detoxification in mice. YB-1 expression was evident in the liver at E14.5, and declined along with liver maturation. Forced expression of YB-1 in fetal liver cells resulted in suppression of CPS1 expression. YB-1 bound to a Y-box in the CPS1 promoter, down-regulating the transcriptional activity induced by C/EBPα. Moreover, CCl_4-induced liver injury up-regulated YB-1 expression, accompanying the suppression of CPS1 and increase of serum ammonia and decrease of urea concentration. ChIP assays showed that YB-1 bound to the CPS1 promoter in fetal and CCl_4-injured livers, but not in normal adult liver in mice. Based on these results, we conclude that YB-1 is a negative regulator of CPS1 transcription.

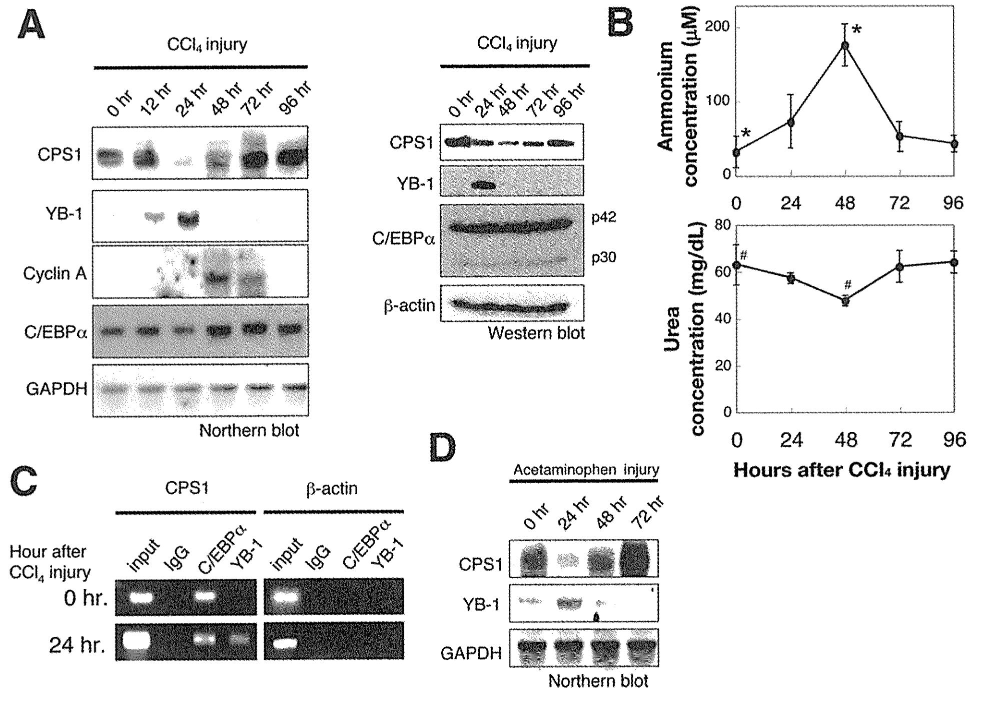

Figure 6. Expression and recruitment of YB-1 in CCl_4-impaired liver. (*A*) Expression of mRNA and protein in CCl_4-induced liver injury. CCl_4 was injected into the peritoneum of mice. RNA and protein were prepared from livers at different time points after the injection. The RNA and protein levels were determined by Northern and Western blot analyses. The level of YB-1 was increased at 24 hours after CCl_4 treatment, whereas the expression of CPS1 RNA decreased to an undetectable level at the same time point. The CPS1 protein level began to decrease at 24 hours after CCl_4 treatment. (*B*) The concentration of ammonia in serum was measured. The level began to rise from 24 hours after CCl_4 treatment, and reached a peak at 48 hours. The concentration of serum urea was measured. The level reached the lowest point at 48 hours. Error bars represent + standard deviations. $N = 4–6$. $*P < .003$; $\#P < .002$. (*C*) Recruitment of C/EBPα and YB-1 to the target DNA demonstrated by chromatin immunoprecipitation assays. (*D*) Acetaminophen was injected into the peritoneum of mice and RNA was prepared from liver at different time points after the injection. RNA levels were determined by Northern blot analysis.

YB-1 Regulates CPS1 Expression

CPS1 is a key enzyme for the urea cycle and CPS1-deficient mice die with hyperammonemia.[4] A deficiency of CPS1 in humans also causes hyperammonemia that results in complications including developmental delay and mental retardation.[1] Thus, CPS1 is essential for ammonia detoxification and its expression requires C/EBPα.[9] CPS1 is not expressed in fetal liver and its expression requires C/EBPα in neonatal liver. Previous studies showed that CPS1 is regulated by several transcription factors, such as C/EBPα, HNF3, and GR.[6,26] The distal −6.3-kb enhancer and the proximal promoter of the *CPS1* gene are important for CPS1 expression. The GRE site is present in each region and has been considered to bridge the 2 regions via the GR–GR interaction.[8] Consistently with the previous studies we show that the −6.3-kb enhancer increases promoter activity induced by C/EBPα. This study has revealed that a Y-box is present only 10 nucleotides from the C/EBP-binding site in the CPS1 promoter and partially overlaps with the GRE (Figure 4*A*). Mutation analysis showed that the inhibition of C/EBPα-dependent luciferase expression by YB-1 was in fact mediated by this Y-box (Figure 4*B*; Supplementary Figure 3*B*). The Y-box we identified is consistent with the repressive element I in the CPS1 promoter, which was previously found in rat hepatoma cells.[30] YB-1 inhibits transcription from pGL4-CPS1 (proximal)-Luc as well as pGL4-CPS1 (distal-proximal)-Luc, indicating that YB-1 suppresses the cooperative transcription activation by C/EBPα and GR as well as C/EBPα alone. ChIP assays also indicated that YB-1 binds to the CPS1 promoter in both mouse fetal liver and regenerating liver damaged by administration of CCl_4, but not in normal adult liver (Figure 6*C*). These results strongly suggest that YB-1 physically interferes with the formation of transcription complex, which contains the transcription factors on the promoter and the distal enhancer.

The expression patterns of several metabolic genes, such as CPS1, phosphoenolpyruvate carboxykinase (PEPCK), TAT, and G6Pase during development, are very similar; in fact, their expression requires C/EBPα. However, each gene seems to be regulated by an additional mechanism. Expression of TAT and G6Pase also requires C/EBPα and putative Y-box sites are present in their 5′ untranslated regions; however, their expression was not affected by YB-1 (Figure 2*A*). Because their Y-boxes are far from the C/EBPα binding site in their promoters, the distance between the YB-1 and C/EBPα binding sites may be an important factor for YB-1 to inhibit C/EBPα function. In the case of PEPCK and G6Pase, their expression is also cooperatively regulated by C/EBPα and Foxo1, a target of insulin signaling.[21] Posttranslational modifications of the liver enriched transcription factors also affect the gene expression, such as SUMOylation of C/EBPα, is also considered to suppress TAT promoter transcriptional activity.[31] Thus, in addition to C/EBPα, several distinct mechanisms are involved in expression of metabolic genes during development.

Role of YB-1 in Liver Development and Injury

Immature hepatocytes proliferate vigorously in fetal liver and differentiate into mature hepatocytes around the time of birth. YB-1 is expressed in fetal liver and HCC, indicating a positive correlation between YB-1 expression and cell proliferation.[15,17,32] We showed that YB-1 and cyclin A were highly expressed in E14.5 mouse liver and disappeared with differentiation (Figure 1*A*). Although a weak signal for YB-1 mRNA was detected even after birth, YB-1 protein was not detected in normal adult liver by Western blotting (Figure 1*B*), suggesting there to be posttranscriptional or posttranslational regulation of YB-1 in the adult liver. The absence of YB-1 protein in normal liver is also supported by the ChIP assays. YB-1 was not expressed in adult liver, but was re-expressed in regenerating liver injured by the administration of CCl_4 or acetaminophen (Figure 6*A* and *D*).

The expression of YB-1 was shown to be up-regulated by c-Myc in cancer cells,[33] consistent with the co-expression of c-Myc and YB-1 in fetal hepatocytes and also HCC.[34] In CCl_4-injured liver, c-Myc expression is also up-regulated at an early phase.[35] Thus, YB-1 may be involved in the network of c-Myc–mediated signaling in liver. YB-1 was also shown to increase the expression of PTP1B, a protein tyrosine phosphatase, to regulate signaling pathways triggered by cytokines, growth factors, and hormones. The expression of antisense YB-1 in Rat1 cells enhanced gp130-mediated signaling,[36] suggesting YB-1 to play a role in the suppression of gp130-mediated signaling. Because gp130 is important for the expression

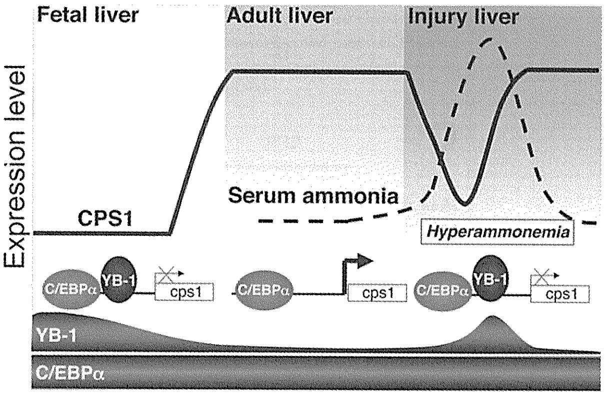

Figure 7. Role of YB-1 in CPS1 expression and ammonia detoxification. YB-1 is highly expressed in fetal liver and down-regulated along with differentiation, whereas C/EBPα is constitutively expressed. YB-1 is absent in normal adult liver, but is expressed in injured liver. In contrast, CPS1 is absent in fetal liver and expressed in normal liver. CPS1 expression is down-regulated in injured liver, accompanying hyperammonemia.

of liver functions,[18] the shutdown of YB-1 expression in the liver late in gestation may be necessary for gp130-mediated expression of various metabolic functions at the perinatal and postnatal stages. Taken together, these results strongly implicate YB-1 in the proliferation and also maturation of hepatocytes.

In conclusion, this study reveals that YB-1 regulates ammonia metabolism by modulating transcription of the CPS1 gene and solves a puzzle on CPS1 expression; although C/EBPα is essential for the CPS1 expression and is constitutively expressed in the liver throughout its development, CPS1 is not expressed in fetal liver (Figure 7).

Supplementary Data

Note: To access the supplementary material accompanying this article visit the online version of *Gastroenterology* at www.gastrojournal.org and at doi: 10.1053/j.gastro.2009.02.064.

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Conflicts of Interest

The authors disclose no conflicts.

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Inducible expression of *Wnt* genes during adult hepatic stem/progenitor cell response

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ABSTRACT

In injured livers where hepatocyte growth is severely limited, facultative hepatic stem/progenitor cells, termed oval cells in rodents, are known to emerge and contribute to the regeneration process. Here, we investigated a possible involvement of Wnt signaling during mouse oval cell response and found significant upregulation of several *Wnt* genes including *Wnt7a*, *Wnt7b*, and *Wnt10a*. Accordingly, increase of β-catenin protein was observed in oval cell compartments. Pharmacological activation of the canonical Wnt/β-catenin signaling induced proliferation of cultured hepatic stem/progenitor cell lines. These results together implicate the role of Wnt/β-catenin signaling in adult hepatic stem/progenitor cell response.

1. Introduction

The liver possesses a unique and remarkable capacity to regenerate upon various injuries, such as those caused by partial hepatectomy or toxic insults. The liver regeneration can usually be achieved by proliferation of the differentiated postmitotic hepatocytes that remain intact, without necessitating an involvement of stem/progenitor cell populations [1]. However, under the severe and/or chronic liver damage conditions where hepatocyte proliferation is suppressed, the facultative stem/progenitor cells are known to emerge and contribute to the regeneration process. Those stem/progenitor cells, referred to as oval cells in rodents, are characterized by their potentials to proliferate as well as to differentiate into both hepatocytes and cholangiocytes, the two epithelial lineages in the liver [2–5]. Despite of their functional relevance in the liver pathophysiology being implicated, the nature and the regulatory mechanisms of the adult hepatic stem/progenitor cells still remain largely unclear.

The Wnt family of secreted factors plays multiple critical roles in regulation of various aspects of liver biology. The Wnt ligands can activate multiple signaling pathways in their target cells, among which the canonical pathway mediated by β-catenin is the best characterized and also highly relevant in stem cell regulation. In the canonical pathway, binding of Wnts to the receptor Frizzled (Fzd) inhibits the kinase GSK3β that normally phosphorylates and primes destruction of the cytoplasmic β-catenin in the absence of ligands, thereby leading to stabilization of the β-catenin proteins, which in turn translocate into the nucleus and mediate transcriptional activation of target genes by forming a complex with the DNA-binding factor TCF/LEF [6,7]. In addition to its role in adult hepatocytes, including liver zonation and hepatic carcinogenesis, the relationship of Wnt/β-catenin signaling with fetal liver stem/progenitor cells in particular have been extensively studied to date and established critical roles of this signaling pathway in controlling proliferation, differentiation, and self-renewal of these cells (reviewed in [8], and references therein; [9]). Recently, activation and possible functional involvement of the Wnt/β-catenin pathway in adult liver stem/progenitor cells have also been implicated by two groups using rodent models of oval cell induction [10,11]. However, detailed time course of its activation with reference to the kinetics of the stem/progenitor cell response was not clarified. Moreover, direct effect of the pathway activation on the adult stem/progenitor cell biology has so far been tested using only one particular cell line.

In this study, we also explored a possible involvement of Wnt/β-catenin signaling in regulation of adult hepatic stem/progenitor cells by employing a mouse oval cell induction model, where administration of the hepatotoxin 3,5-dietoxycarbonyl-1,4-dihydro-collidine (DDC) causes severe chronic liver injury and stimulates emergence and massive proliferation of oval cells [12].

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2. Materials and methods

2.1. Antibodies and reagents

Polyclonal rabbit anti-CK19 antibody was raised as previously described [13]. Polyclonal rabbit anti-β-catenin antibody and monoclonal mouse anti-β-catenin antibody were obtained from SIGMA and BD Transduction Laboratories, respectively. The GSK3*β* inhibitor 6-bromoindirubin-3′-oxime (BIO) [14] was kindly synthesized and provided by Drs. Aya Tanatani and Yuichi Hashimoto (IMCB, The University of Tokyo). The inactive analog 1-methyl-BIO (MeBIO, or GSK-3 inhibitor XIV; Calbiochem) was used as a negative control.

2.2. Mice and oval cell induction

C57BL/6 mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) and maintained under a standard SPF condition. All animal experiments were performed with procedures according to the guideline set by the institutional animal care and use committee of the University of Tokyo. Male mice of 8–12 week old were fed 0.1% DDC-containing diet (F-4643; bio serve) to induce hepatic oval cell response, and then killed to harvest liver samples.

2.3. RNA preparation and cDNA synthesis

Total RNA was prepared from whole liver samples homogenized in TRIzol reagent (Invitrogen), treated with DNaseI (Invitrogen), and then used for cDNA synthesis using SuperScript III (Invitrogen) with random hexamer primers.

2.4. PCR analyses

Standard PCR reactions were performed with Blend Taq (TOYOBO), and the products were run in 1.5% agarose gels and visualized with EtBr staining. Quantitative PCR analyses were done using LightCycler (Roche) with SYBR Premix Ex Taq reagent (TaKaRa). *Gapdh* was used as an internal control. The primers used are summarized in Supplementary data.

2.5. Immunohistochemistry

The fresh liver samples were fixed in Zamboni's fixative solution and embedded in OCT compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan). The samples were frozen and sectioned onto APS-coated glass slides (Matsunami Glass Ind. Ltd., Osaka, Japan). After blocking in 5% skim milk/PBS, the samples were incubated with primary antibodies and then with fluorescence-conjugated secondary antibodies. Nuclei were counterstained with Hoechst 33342 (Sigma).

2.6. Cell culture and proliferation assay

The hepatic stem/progenitor cell lines, HSCEs, as well as their precedent bulk culture of the EpCAM$^+$ cell-derived fraction, were maintained in type I collagen-coated dishes using a medium supplemented with 10 ng/ml each of recombinant human EGF and HGF (see Supplementary data). The detail for their establishment and characterization will be described elsewhere (Okabe et al., submitted).

Proliferative response of HSCE cells was examined by a colorimetric assay using WST-1 cell proliferation reagent (Roche)

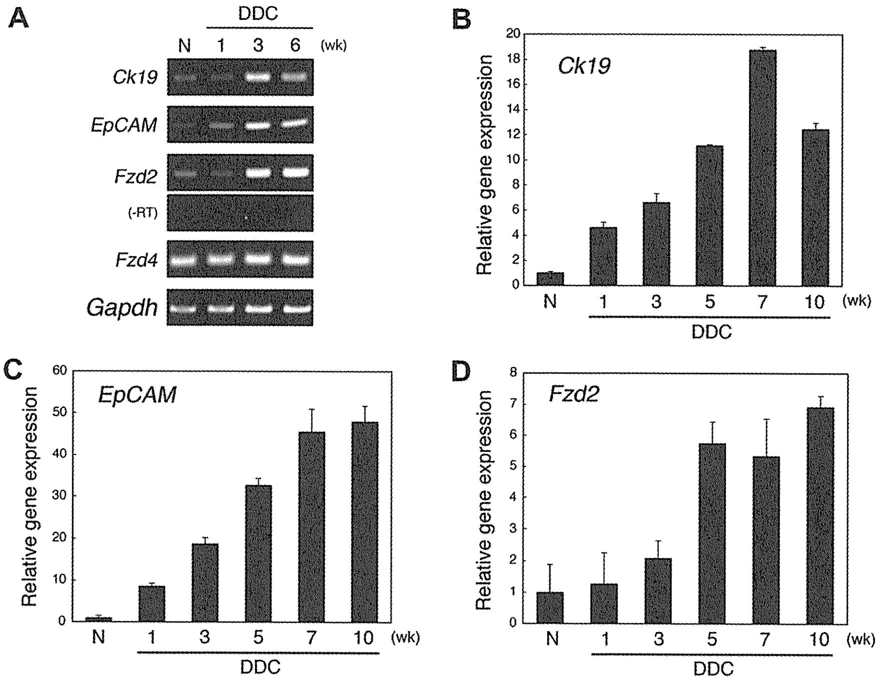

Fig. 1. Expression of *Fzd2* is upregulated in the livers of DDC diet-fed mice concomitantly with the oval cell marker genes. (A) Total RNA was isolated from whole liver samples of normal diet-fed (N) and DDC diet-fed mice, reverse-transcribed, and subjected to PCR analyses to determine expression of *Fzd2*, *Fzd4*, the oval cell markers *Ck19* and *EpCAM*, and *Gapdh*. Note that the primer sets used to detect *Fzd4*, *Ck19*, *EpCAM*, and *Gapdh* were designed to flank one or more intron(s). For the Fzd2 gene, which comprises only of one exon, control RNA samples without reverse transcription (-RT) were also examined, confirming no adverse amplification caused by contaminated genomic DNA. (B) Inducible expression of *Fzd2*, *Ck19*, and *EpCAM* were analyzed by quantitative PCR analyses. Expression was normalized to that of *Gapdh*.

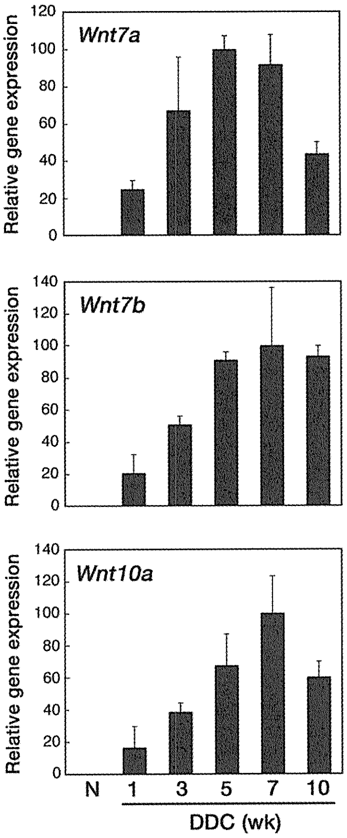

Fig. 2. Inducible expression of *Wnt7a*, *Wnt7b*, and *Wnt10a* in the livers of DDC diet-fed mice. Expression of *Wnt7a*, *Wnt7b*, and *Wnt10a* during the course of oval cell induction was analyzed by quantitative PCR analyses. Expression was normalized to that of *Gapdh*.

according to the manufacturer's protocol. The absorbance value (OD_{450}–OD_{650}) was measured using an Emax microplate reader (Molecular Devices).

2.7. Transfection and luciferase assay

HSCE5 cells were transfected with the TOPtkLuciferase or FOPtkLuciferase plasmid using Lipofectamine with Plus reagent (Invitrogen), and cultured in the presence or absence of the GSK3 inhibitor BIO for 48 h. The cells were lysed in Passive Lysis Buffer (Promega) and subjected to a luciferase assay using a luminometer (MICROLUMAT LB96P; Berthold) with Dual luciferase assay reagent (Promega).

3. Results and discussion

We recently performed a screening project that aimed to isolate cell surface molecules expressed in oval cells, and identified EpCAM as a novel marker for mouse oval cells (Okabe et al., submitted). Notably, EpCAM was recently reported to be a marker for rat oval cells [15]. In the same screening, we also noticed two Frizzled (Fzd) family members, Fzd2 and Fzd4, as molecules expressed in the oval cell-induced rat livers. To explore the possible involvement of these Fzd molecules in oval cell biology, we examined their expression in the mouse DDC diet model for oval cell induction. As shown in Fig. 1A, feeding with DDC diet resulted in oval cell induction in mice, as manifested by strong upregulation of the oval cell marker genes *CK19* as well as *EpCAM*. Basal expression of these genes in the normal liver derived from cholangiocytes, which are known to express these markers as well. During the course of oval cell induction, *Fzd2* also was strongly upregulated. Expression of *Fzd4*, although apparently observed in DDC diet-fed samples, was not inducible but rather constant. Quantitative PCR analyses further confirmed that *Fzd2*, like *CK19* and *EpCAM*, was highly upregulated in oval cell-induced mouse livers (Fig. 1B–D).

The Frizzled family molecules including Fzd2 function as the receptor component for the Wnt family ligands, which prompted us to investigate possible induction of the *Wnt* genes in the oval cell-induced livers. We compared expression of all the 19 members of the mouse *Wnt* genes and found that several of them were significantly upregulated in the livers of DDC diet-fed mice (Fig. S1A). Specifically, *Wnt7a*, *Wnt7b*, and *Wnt10a* showed strong upregulation with more than 50-fold increase above the basal level in the normal livers. We further examined the time course of expression of those *Wnt* genes and confirmed that their induction kinetics was well correlated with that of the oval cell appearance (Fig. 2; com-

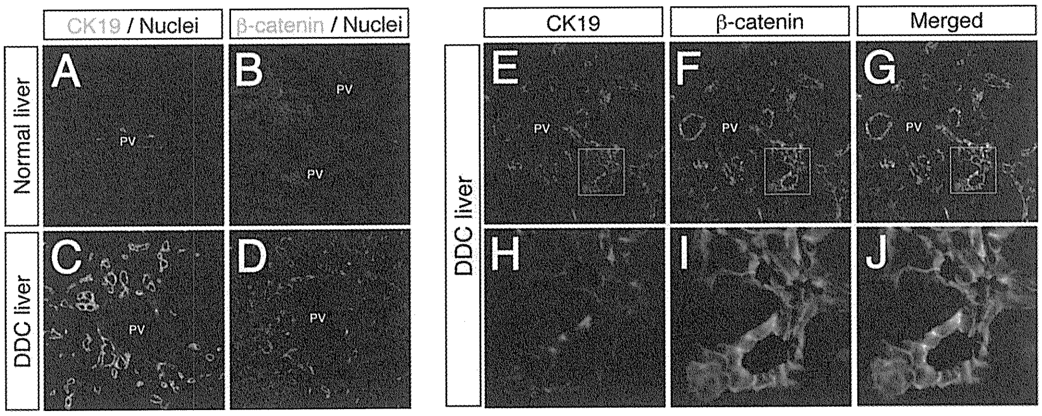

Fig. 3. β-catenin proteins are increased in oval cells. A–D. Liver sections prepared from a normal control mouse (A and B) and a DDC diet-fed mouse (5 wk; C and D) were subjected to immunofluorescent staining analyses using anti-CK19 (A and C) and anti-β-catenin (B and D) antibodies (Green). Nuclei were counterstained with Hoechst 33342 (Blue). E–J. Double staining experiments were performed to confirm co-expression of CK19 (E and H) and β-catenin (F and I) in the livers of the oval cell-induced mice. Panels G and J shows the merged images, where nuclear staining by Hoechst 33342 is also included (Blue). Panels H–J provide higher power images corresponding to the squared regions in panels E–G, respectively. PV, portal vein.

pare with Fig. 1B and C). For comparison, we also monitored expression kinetics of *Wnt3* and *Wnt3a*, two representative members of the canonical Wnt ligands, and found that neither of them was expressed during the course of oval cell induction up to 10 weeks.

It has been reported that Wnt7a, Wnt7b, and Wnt10a are all capable of activating the canonical signaling pathway [16–19]. As a well-established hallmark of the canonical Wnt signal activation is stabilization and accumulation of β-catenin, we performed immunostaining analyses of this molecule using liver sections (Fig. 3). β-Catenin is a component of the cell adhesion complex in every hepatocyte, so that staining of the normal liver samples detected its basal level expression with a relatively uniform distribution pattern (Fig. 3B). In damaged livers, oval cells are known to emerge from periportal regions, forming duct-like structures, which was readily detectable based on expression of the marker molecule CK19 (Fig. 3C). Remarkably, those propagating oval cells were strongly positive for anti-β-catenin immunostaining (Fig. 3D). Double staining experiments confirmed that the intense β-catenin signals indeed co-localized with CK19 expression in the same cells (Fig. 3E–J). Although the signals appeared most intense at and adjacent to the cell membrane, weaker yet significant signals were also observed diffusively throughout the cytoplasm as well as in the nucleus in some if not all cells. It should be noted that, in many cases, cells that undergo Wnt signaling may display an overall rise in β-catenin protein without a clear nuclear preference [6]. These results suggest that the induced expression of Wnt ligands leads to concomitant activation of the canonical signaling pathway in oval cells.

The canonical Wnt signaling stimulates transcriptional activation of various target genes. To further confirm that the canonical pathway is indeed turned on and functioning in oval cells, we assessed induction of known target genes in the oval cell-induced livers. Using fractionated cell samples derived from the livers of DDC diet-fed mice, we observed that several of the known targets, including *Axin2*, *N-myc*, and *Wisp1*, were significantly upregulated predominantly in the EpCAM$^+$ oval cell population (Fig. S2). Moreover, a recent study has reported that the oval cell marker EpCAM is itself a direct transcriptional target of Wnt/β-catenin pathway [20]. Together, these facts further support the notion that the canonical pathway is functionally activated in oval cells.

To gain an insight into the role that active Wnt/β-catenin signaling plays in oval cell regulation, we examined whether this could affect proliferation of hepatic stem/progenitor cells *in vitro*. The bi-potential adult hepatic stem/progenitor cell lines, referred to as HSCEs, were originally established from the EpCAM$^+$ oval cell fraction in the livers of DDC diet-fed mice, and is capable of proliferating in the presence of EGF and HGF (Okabe et al., submitted). RT-PCR analyses revealed that a representative clone of HSCE (clone 5; HSCE5) expresses several members of the Fzd family receptors, as well as the co-receptors Lrp5/6 (Fig. S3), suggesting that the cells could respond to Wnt stimulation *per se*. We employed a small compound GSK3*β* inhibitor, BIO, which has been shown to be capable of mimicking activation of the canonical pathway by suppressing GSK3*β*-mediated phosphorylation and concomitant degradation of β-catenin [14]. Stimulation with BIO indeed led to activation of the canonical pathway in HSCE5, as demonstrated by induction of the β-catenin/TCF-dependent TOP-tkLuciferase reporter activity (Fig. 4A). We then tested the effect of BIO on proliferation of HSCE5, and found that application of the compound did induce significant proliferative response of the cells even in the absence of EGF and HGF, to a level nearly comparable to the one induced by these cytokines (Fig. 4B). Application of 5 mM of BIO resulted in a less effect, due presumably to its cyto-

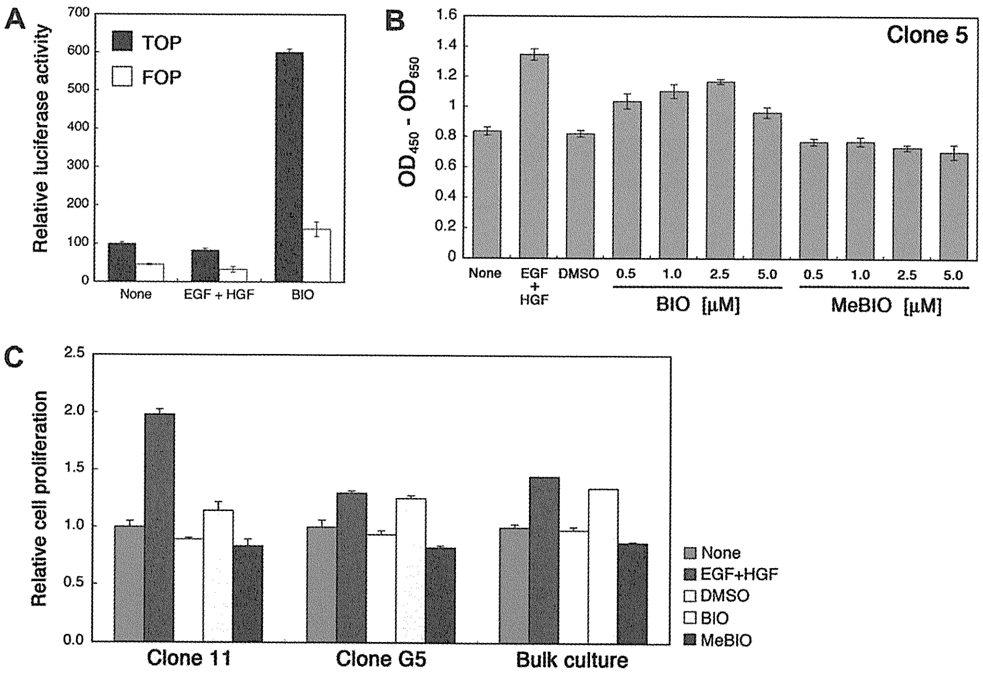

Fig. 4. Pharmacological activation of Wnt/β-catenin signaling stimulates proliferation of the adult hepatic stem/progenitor cell lines HSCE. (A) HSCE5 cells were transfected with TOPtkLuciferase (TOP; a reporter for canonical Wnt signaling activity) or FOPtkLuciferase (FOP; an unresponsive control), cultured in the presence or absence of the GSK3 inhibitor BIO, and then subjected to a luciferase assay. Stimulation with EGF and HGF was used as a control. (B) HSCE5 cells were plated in a 96-well plate at a density of 2.5×10^3 cells/well, and cultured in the presence or absence of BIO, its inactive analog MeBIO, or the vehicle (DMSO). Stimulation with EGF and HGF was used as a control. After 48 hours of incubation, the level of cell proliferation was examined by WST-1 assay. (C) Proliferative response of additional HSCE cell lines (clones 11 and G5), as well as the bulk culture of the EpCAM$^+$ cell fraction-derived cells before being subjected to clonal isolation of HSCE cell lines, were examined by WST-1 assay as in (B). Note that the clone G5 harbors the retrovirally-transduced GFP marker gene.

toxicity at this dose. The inactive analog MeBIO was used as a negative control, showing no obvious effect. Finally, we tested the effect of the compounds on other independent clones of HSCE, as well as on the bulk culture of the EpCAM$^+$ cell fraction-derived cells before being subjected to clonal isolation of HSCE cell lines. As shown in Fig. 4C, all of them were capable of responding to stimulation with BIO, resulting in significant proliferative induction.

In addition to the β-catenin-dependent canonical signaling pathway, some Wnt ligands can activate non-canonical signaling pathways, such as the planar cell polarity/c-Jun N-terminal kinase (JNK) pathway and the Ca^{2+}-mediated pathway [21]. Notably, it has been reported that Wnt7a and Wnt7b can activate the JNK pathway in endometrial cancer cells and hippocampal neurons, respectively [22,23], while Fzd2 has been shown to mediate activation of non-canonical signaling pathways in certain types of cells [21,24–26]. Although our results have clearly established activation of the canonical pathway *in vivo* and its functional effect *in vitro*, a possible involvement of the non-canonical pathways in oval cell regulation cannot be neglected and should also be addressed in future studies.

In summary, our present study has identified several Wnt ligands and the downstream canonical signaling pathway as a possible regulatory signal for mouse oval cells, a well-recognized facultative stem/progenitor cell population in the adult liver. Much progress has been made in recent years in identifying and characterizing various tissue stem cells as well as their specialized surrounding microenvironments, or stem cell niches. The niches play fundamental roles in controlling proliferation, differentiation, and/or self-renewal of the stem cells through direct cell–cell interactions and also via various soluble cytokines, such as Wnts, Hedgehogs, and BMPs [27,28]. Although it still remains unknown whether any niche structures are formed to support hepatic oval cells, it is tempting to speculate that Wnt/β-catenin signaling may play a role as a part of the niche signals in regulating their development and behaviors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.01.022.

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