The alpha-fetoprotein (AFP) gene is highly activated in fetal liver but is dramatically repressed shortly after birth. The mechanisms that underlie AFP transcriptional repression in postpartum liver are not well understood. AFP enhancer, repressor region, and promoter are implicated to be involved in AFP postnatal repression, but the major transcriptional repressor remains undefined. We previously identified a zinc finger protein gene ZBTB20. To determine its physiological functions in vivo, we have generated hepatocyte-specific ZBTB20 knockout mice by the Cre/loxP approach and demonstrated here that ZBTB20 ablation in liver led to dramatic derepression of the AFP gene in entire liver throughout adult life, although the hepatocytes were normally under nonproliferating status. Furthermore, we found that ZBTB20 was a transcriptional repressor capable of specifically inhibiting AFP promoter-driven transcriptional activity. Liver chromatin immunoprecipitation and mobility shift assays showed that ZBTB20 bound to AFP promoter directly. ZBTB20 was developmentally activated in liver after birth and inversely correlated with its AFP gene expression, suggesting that activated ZBTB20 expression in liver mediated AFP gene repression. Our data point to ZBTB20 as a key regulator governing AFP gene transcription and postulate a new model for the postnatal gene repression of AFP in liver.

Results

Generation of Liver-Specific ZBTB20 Knockout Mice. Initially, we identified the BTB zinc finger protein ZBTB20 (also known as DPZF) in human dendritic cells (20). To specifically investigate its functional role in liver, we created liver-specific ZBTB20 knockout mice (LZB20KO). First, we developed mice with loxP sites flanking exon 6 of the ZBTB20 gene (21), which encodes 540 aa of ZBTB20 protein spanning from the complete POZ domain to the first zinc finger domain (21). Each of the mice was confirmed to participate in neurogenesis in a transgenic model (22). However, its physiological functions are not established. In the present study, we generated liver-specific ZBTB20 knockout mice (LZB20KO) and found that ZBTB20 was required for AFP postnatal repression in liver. Our results demonstrate that ZBTB20 is a key transcriptional repressor of the AFP gene in a dominant fashion.
The neo cassette was removed in the male germ line by backcrossing to protamine promoter-Cre expressing transgenic mice (23), which led to the creation of the ZBTB20\textsuperscript{flox} allele (Fig. 1C and D). The ZBTB20\textsuperscript{flox} allele was bred to homozgyous, which did not affect the viability or fertility of these mice, suggesting that the flanking loxP sites did not significantly alter the expression from the targeted locus.

LZB20KO mice were generated by repeated mating of ZBTB20\textsuperscript{flox/+} mice with rat albumin promoter-Cre (ALB-Cre) transgenic mice (24). PCR analysis of genomic DNA showed that exon 6 was efficiently disrupted in LZB20KO liver, which gave a 1.2-kb band and that the residual signal of a 0.8-kb band reflects the un-recombined ZBTB20\textsuperscript{flox} allele, most likely from nonhepatocyte cells in the liver (Fig. 1E). Efficient deletion of the ZBTB20 gene in LZB20KO liver was confirmed at the mRNA level by real-time RT-PCR and at the protein level by immunoblot analysis with ZBTB20-specific antibody (Fig. 1F and G). By contrast, no deletion was detected in the brain, muscle, heart, kidney, or lung from LZB20KO mice. There was no effect on ZBTB20 expression in ZBTB20\textsuperscript{flox/flox} littermates (data not shown).

LZB20KO (ZBTB20\textsuperscript{flox/flox}/ALB-Cre) and control mice (ZBTB20\textsuperscript{flox/flox} and ALB-Cre) were born normally and survived equally into adulthood with no significant difference in development. Both male and female LZB20KO mice were fertile and produced normal-sized litters. The livers from LZB20KO mice were normal in appearance and size, and histological examination revealed no abnormality in their architecture [supporting information (SI) Fig. S1], suggesting that ZBTB20 was dispensable for liver development.

Dysregulation of AFP in Liver. To evaluate the differentiation status of hepatocytes, we analyzed the expression pattern of albumin and AFP. Real-time RT-PCR analysis showed that adult LZB20KO liver expressed very high levels of AFP mRNA, \( \sim 3,000 \)-fold higher.
Than the basal levels of control littermates (Fig. 2A). To distinguish between the failure of shutoff and reactivation after birth that led to the dysregulated expression of AFP in adult Lzb20KO liver, we explored the ontogeny of AFP gene expression during the postnatal development of Zbtb20-deficient liver. Consistent with a previous report (1, 25), AFP mRNA was activated gradually in fetal liver (Fig. S2), declined 10,000-fold to basal levels in wild-type and Lzb20flox/flox liver within four weeks after birth. Whereas, AFP levels in Lzb20KO liver were comparable at birth but decreased 5-fold in the first four weeks of age, which was followed by the high and constitutive expression throughout adult life (Fig. 2A and B and data not shown). Northern blot and western blot analysis also confirmed the abundant expression of AFP in adult Lzb20KO liver (Fig. 2C and D). This indicated that aberrant expression of AFP in the postnatal Lzb20KO liver was due to the failure of transcriptional shutoff. On the other hand, Lzb20KO mice from either young (2-week-old) or adult (20-week-old) mice expressed comparable levels of ALB mRNA (Fig. 2C). These data suggest that AFP postnatal dysregulation in Lzb20KO liver was gene-specific, supporting the current view on the autonomous regulation of AFP and ALB gene expression (1).

We further examined the zonal expression pattern of AFP in the Lzb20KO liver. To this end, we took advantage of the AFP mutant mice harboring the LacZ reporter gene in the AFP locus under the control of AFP P1 promoter (2), which allowed us to assess the transcriptional activity of the AFP gene by X-Gal staining. Consistent with nearly complete repression of AFP gene transcription, β-galactosidase was undetectable in the adult liver from AFP single mutant mice (Fig. 2E). However, robust β-galactosidase activity was detected throughout the adult livers from Zbtb20flox/flox/Alb-cre/AFP+/- compound knockout mice (F) rather than the same-aged Zbtb20flox/flox/Alb-cre/AFP+/- knockouts (E), which reflected the AFP gene transcriptional activity. Error bar represents SD.

**Fig. 2.** Dysregulated AFP expression in Lzb20KO liver. (A) AFP mRNA expression in liver from 4-month-old control (gray bar) and Lzb20KO (black bar) mice was shown by real-time RT-PCR. n = 3 experiments. (B) Real-time RT-PCR analysis for AFP mRNA expression in liver from control (gray bar) and Lzb20KO (black bar) mice during postnatal days 1–28. Each group at individual time points included at least 5–8 mice. (C) Northern blot analysis for AFP and ALB mRNA expression in the livers from 1-day-, 2-week- and 20-week-old Lzb20KO mice (lanes 2, 4, and 6) and their control littermates (lanes 1, 3, and 5). The RNA loading control was demonstrated by EtBr staining of the gel. (D) Western blot analysis of AFP expression in whole liver lysates from 3-week-old Lzb20KO and control littermates (lanes 1, 3, and 5). The RNA loading control was demonstrated by reprobing with anti-actin antibody. (E and F) In situ β-galactosidase staining showed LacZ expression throughout the liver from 3-week-old Zbtb20flox/flox/Alb-cre/AFP+/- compound knockout mice (F) rather than the same-aged Zbtb20flox/flox/Alb-cre/AFP+/- knockouts (E), which reflected the AFP gene transcriptional activity. Error bar represents SD.
analyzed the effects of ZBTB20 on their activity (Fig. 4D). Serial deletion of the fragment from –2309 to –163 of the AFP gene did not significantly alter the repressive effects of ZBTB20 on the resultant AFP reporters, suggesting that the ZBTB20-responsive region might be located in the –162 to –27 region of the AFP promoter. The effort to analyze the effects of ZBTB20 by further 5′ deletions of the AFP gene was hampered by their weak reporter activity. These data suggest that the minimal AFP promoter (–162/+27) contains a cis-acting element mediating ZBTB20 repressive activity.

Binding of ZBTB20 to AFP Promoter. To determine whether ZBTB20 binds directly to DNA in the AFP promoter, we performed gel mobility shift assay using recombinant GST fusion protein of ZBTB20 zinc finger domain (531–741aa). Double-stranded oligonucleotides corresponding to –108 to –53 of mouse Afp gene bound to ZBTB20 with high specificity, rather than the fragment –172 to –104 or –56 to +27 (Fig. 5A and data not shown). The DNA-protein complex was supershifted with anti-ZBTB20 antibodies and was abolished by excess unlabeled DNA fragment –108 to –53 as a competitor (Fig. 5B), indicating that ZBTB20 could bind to the AFP promoter directly at –108 to –53 (Fig. S3).

To determine whether endogenous ZBTB20 interacts with the AFP promoter in AFP postnatal repression, we performed a chromatin immunoprecipitation (ChIP) assay in adult liver, where ZBTB20 is highly expressed and AFP is fully repressed. We found that ZBTB20 was bound at the AFP promoter in normal adult liver (Fig. 5 C and D). With histone H3 as a positive control for chromatin recovery, no ZBTB20 binding was detected at a control distal genomic locus, the 18S ORF, indicating that association of ZBTB20 to the AFP promoter was gene-specific.

Developmental Expression of ZBTB20 and Postnatal Repression of AFP Transcription in Liver. Last, we analyzed the expression pattern of ZBTB20 in liver development. Immunostaining showed that ZBTB20 was a nuclear protein nearly uniformly detectable in most hepatocytes from adult WT liver (Fig. 6A), whereas no ZBTB20 staining was detected in the hepatocytes from LZB20KO mice (Fig. S4). RT-PCR analysis demonstrated that ZBTB20 mRNA was poor in fetal liver (data not shown) and developmentally activated in postpartum liver with a 4-fold increase at the age of two weeks, a 12-fold increase at the age of 8 weeks, and after that reaching a plateau (Fig. 6B). Western blot analysis revealed that ZBTB20 protein was undetectable in the nuclear extracts from the neonatal liver and elevated significantly during postnatal development (Fig. 6C). We detected two distinct isoforms of ZBTB20 in liver, which are likely produced by alternative utilization of the start codon in translation (21). In the early stage of postnatal development, the long one was dominant in liver. However, the short one became dominant after four weeks of age.

Taken together, we postulate a model for postnatal repression of AFP gene transcription in liver. In this model, ZBTB20 is developmentally up-regulated in postpartum liver and acts as a dominant transcriptional repressor by interacting with the AFP promoter to repress AFP gene transcription to basal levels (Fig. 6D). Ablation of ZBTB20 in postpartum liver leads to derepressed aberrant expression of AFP gene independent of hepatocyte proliferation.

Discussion

Our results establish zinc finger protein ZBTB20 as a key repressor governing AFP gene transcription in postpartum liver. First, ex-
pression of AFP and ZBTB20 is developmentally regulated and exhibits an inverse correlation with each other in postpartum liver, where ZBTB20 is gradually activated while AFP is dramatically repressed to extremely low levels. Moreover, our unpublished data show that ZBTB20 expression was decreased in chemically induced rodent hepatoma, where AFP was reactivated. Second, hepatocyte-specific disruption of ZBTB20 gene in mice results in dramatic derepression of AFP in the entire liver throughout adult life with expression levels almost close to fetal liver. Third, forced expression of ZBTB20 represses AFP-driven transcriptional activity in vitro. Last, ZBTB20 binds to the AFP promoter both in vitro and in vivo, which mediates postnatal repression of AFP transcription.

AFP gene transcription is regulated by positively and negatively acting factors that bind to specific elements in different regions of the AFP gene. Hepatocyte-enriched transcriptional activators, such as HNF1, HNF3, and C/EBP, are thought to play critical roles in AFP activation in a tissue-specific manner. On the contrary, transcriptional repressors involved in AFP gene regulation are poorly defined. There is a DNA-binding site located at −135 of the AFP promoter for transcriptional repressor COUP-TF (26, 27), but its physiological significance in regulation of AFP transcription is still unknown. Overexpression of c-Jun in hepatoma cells inhibits AFP promoter activity in a DNA binding-independent manner (28). Also, p53 mediates AFP repression by competing with HNF3 to bind DNA in the repressor region of the AFP gene (−838 to −250). The p53-null mice display a delay in AFP postnatal repression in liver with eventual repression at four months of age (15). The Zbtb20 gene, which encodes a zinc finger and homeobox protein, regulates AFP postnatal repression in liver, and its mutation in BALB/cJ mice leads to 5–20-fold higher adult serum AFP levels (29). Enhancer III participates in AFP postnatal repression (16), but the involved transacting repressor remains undefined. Our finding that ZBTB20 interacts with the AFP promoter and represses AFP gene transcription in a dominant fashion sheds light on the physiological nature of AFP gene regulation.

The most likely scenario is that ZBTB20 interferes with transcriptional stimulation by activators or recruits corepressors to inhibit transcription and/or remodel chromatin structure. Transgenic studies show EIIH may be responsible for postnatal repression of AFP transcription (16). It will be worthwhile to examine whether ZBTB20 is involved in EIIH-mediated repression or whether there is synergy between these distinct repression mechanisms. We note that there is also a several-fold decrease of AFP mRNA in adult ZBTB20-null liver compared with fetal liver, suggesting that other mechanisms also play important roles in AFP postnatal repression. It is possible that postnatal repression in AFP-null liver is mediated by other identified (e.g., p53, Zbt2, and COUP-TF) or unidentified transcriptional repressors. Less likely, it may result from the reduction of transcriptional activators. We are further investigating the potential repression mechanisms of ZBTB20 and the mechanisms of AFP repression in ZBTB20-deficient liver. We speculate that multiple factors and multiple mechanisms may be involved in complete repression of AFP gene in postpartum liver.

The relation between liver cell proliferation and AFP gene transcription has been noted but not fully addressed yet. Postnatal AFP repression in liver is coincident with the cessation of hepatocyte cell division, and AFP reactivation is often related to the hepatocyte proliferation (e.g., regeneration and carcinogenesis) (1). It is thought that there may be a mechanistic link between this temporal correlation, which is further supported by in vitro studies of AFP reactivation (30). However, the adult Lzbt2KO liver has a normal architecture and remains in a normally nonproliferating status, despite their aberrant expression of AFP. This raises the possibility that AFP expression is independent of cell proliferation in the absence of ZBTB20. Furthermore, although not essential in the absence of ZBTB20, aberrant proliferation may facilitate loss of ZBTB20 interaction with chromatin to promote AFP derepression during HCC or liver regeneration. Nevertheless, it should be interesting to determine whether AFP reactivation in HCC reflects the decreased levels or activity of ZBTB20. Our unpublished data demonstrated that ZBTB20 expression was down-regulated in diethylamino-oxide-induced mouse HCC, where AFP was markedly reactivated. Whether the AFP derepression in ZBTB20-deficient hepatocytes implies the potentially enhanced susceptibility of hepatocellular carcinogenesis is under examination.

Taken together, our identification of ZBTB20 as a dominant repressor of AFP gene transcription provides insight into AFP gene regulation, which will help to unravel the biochemical basis of AFP reactivation in carcinogenesis and facilitate earlier diagnosis of a disease that is generally untreatable by the time of its discovery.

Materials and Methods

Generation of Lzbt2KO Mice. The targeted ES cells carrying the Zbtb20neo-flx allele were injected into C57BL/6 blastocysts to produce chimeric mice, which transmitted the Zbtb20neo-flx allele to the progeny. The neomycin resistance cassette was removed by breeding with protamine promoter-cre transgenic mice. Liver-specific ZBTB20 knockout mice were generated by crossing Zbtb20flx mice with Alb-Cre transgenic mice. See Si Methods for detail. Lzb20KO mice were repeatedly crossed to AFP-deficient mice (AFPtm1tm1, C57BL/6 background), which harbor the LacZ gene in the AFP mutant allele driven by the AFP promoter (2). Animal experiments were done following institutional guidelines.

Expression Plasmids. Full-length mouse ZBTB20 cDNA encoding 741 aa was cloned from liver by RT-PCR and inserted into the expression vectors pcDNA3 (Invitrogen) and pCMV-FA (Stratagen). In the latter, it was in-frame fused with Gal4-DBD (1–147 aa) at the N terminus. The fragment containing five Gal4 binding sites was subcloned upstream of the SV40 promoter in the pGL2-promoter vector (Promega). The mouse AFP promoter with different lengths of 5’ flanking sequence was removed from the plasmid pUC19-AFP (18) and inserted into the pGL3-basic vector (Promega). The mouse GCK promoter (−980 to +1) and ALB promoter (−790 to +23) were cloned by PCR and inserted into the pGL3-basic vector.

Analysis of RNA. Total RNA was extracted from the TRizol (Invitrogen) homogenates. Total RNA (20 μg per lane) were loaded on a 1.2% formaldehyde agarose gel. The RNA was then transferred to a nylon membrane and probed with a 32P-end-labeled synthetic oligonucleotide.
gel and transferred to a nylon membrane. The membranes were hybridized with 32P-labeled mouse AFP or ALB cDNA probes. Real-time RT-PCR was performed in a two-step reaction. First strand cDNA was synthesized with a SuperScript III RT-PCR kit (Invitrogen), and the second step was performed in a fluorescent temperature cycler (Mastercycler ep realplex, Eppendorf) with SYBR green and specific primers for each of the genes. Every plate included the 36B4 gene as an internal control. Primer sequences are available on request. Results were analyzed with the Student’s unpaired t test.

Western Blot Analysis. Liver nuclear extracts were prepared by sucrose gradient centrifugation of homogenates and dissolved in buffer containing 20 mM Hepes (pH 7.9), 1.5 mM MgCl2, 410 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5% Nonidet P-40, 0.5 mM DTT, 0.5 mM PMSF, 4 μg/ml leupeptin, 4 μg/ml aprotinin, and 4 μg/ml pepstatin. We resolved lysates (100 μg of protein per lane) with SDS/PAGE and immunoblotted membranes with anti-ZBTB20 antibodies, rabbit anti-AFP (2), or rabbit anti-Sp1 antibodies (Santa Cruz Biotechnologies).

Chromatin Immunoprecipitation Assay. Liver tissue was isolated from normal adult mice and flash-frozen in liquid nitrogen. ChIP assays of liver tissue were performed as described (15), with minimal modifications. The fragmented, pre-cleared chromatin lysate was incubated overnight with specific antibodies for ChIP; histone H3 (Abcam), ZBTB20, and normal rabbit IgG (Upstate). To analyze specific, antibody- and protein-bound DNA, conventional PCR and quantitative real-time PCR were performed. Conventional PCR primers were generated to detect the AFP core promoter region (∼82–194) as described (15). Real-time PCR was conducted in a 7500 FAST ABI instrument.

Gel Mobility Shift Assay. The double-stranded oligonucleotides from the AFP promoter were labeled with [32P]ATP by T4 polynucleotide kinase, purified with a G25 DNA purification column, and used for the mobility shift assay. The 20-μl binding reaction contained 10 mM Hepes (pH 7.6), 50 mM NaCl, 5 mM MgCl2, 10 mM EDTA, 1 mM DTT, 5% glycerol, 1 μg of poly (dI-dC) (Amersham), 50,000 cpm probes, and 4 ng of GST-fusion protein. After incubation at room temperature for 15 min, the reactions were separated on a 5% PAGE gel containing 0.5× TBE. Excessive unlabeled oligonucleotides were used as competitors, and 2 μg of anti-ZBTB20 antibodies were added in the binding reaction for the supershift assay.

Reporters Assay. Human hepatoma cells ( HepG2 ) were transiently transfected in 24-well plates with plasmids by using Effectene (Qiagen). Forty-eight hours after transfection, cells were disrupted with passive lysis buffer (Promega) and subjected to a luminescent assay in a luminometer (MiniLumat LB9506, Berthold GmbH). SV40-Renilla luciferase plasmid or PMCV-Jβ-Gal was used as an internal control to normalize the luciferase activity, and the β-galactosidase activity was measured by using a luminiscence detection kit (Clontech). The fold repression of transcription was calculated relative to transcription of the reporters in the presence of the relevant empty expression vector and normalized to the internal control. Expression of all of the transfected proteins was confirmed by immunoblotting with the appropriate antibodies.

ACKNOWLEDGMENTS. We thank B. Spear, H. Nakabayashi, and J. Danan for the plasmids and X. Ma and G. Gu for technical assistance. This work was supported by the National Natural Science Foundation of China Grants 90608026 (to W.Z.), and 30772478 (to Z.X.). National “973” Program of China Grant 2006CB503910 (to W.Z.), National “863” Program of China Grant 2007AA02Z173 (to W.Z.), and fellowships from the Ministry of Education of China (to W.Z.), Shanghai Municipal Science and Technology Commission, and Education Commission (to W.Z.).