much weaker compared with the induction of other nuclear IκBs (Fig. S2*A*). These results indicate that, in contrast to the mRNA of other IκBs, IκBη mRNA is constitutively expressed at a basal level and marginally up-regulated by the TLR signaling pathways in macrophages.

IκB proteins can be categorized into two groups. Canonical IκB proteins, like IκBα and IκBβ, are predominantly localized in the cytoplasm and block the nuclear translocation of NF-κB (1). On the other hand, nuclear IκB proteins, such as IκBNS and IκBζ, are present in the nucleus and regulate NF-κB's transcriptional activity (7, 9, 14). To determine the subcellular distribution of IκBη, NIH 3T3 mouse fibroblasts were transfected with cDNA encoding a FLAG-tagged IκBη, and the protein's localization was examined by immunofluorescence staining. We found that FLAG-IκBη as well as endogenous IκBη was predominantly located in the nucleus (Fig. 2 *B* and *C*). To further examine the expression and distribution of IκBη in LPS-stimulated macrophages, we next separated cytosolic and nuclear fractions of Raw264.7 cells and performed Western blotting (Fig. 2*D*). The results showed that NF-κB p65 was translocated from the cytoplasm to the nucleus in response to LPS and stayed in the nucleus for 3 h after the stimulation. However, LPS did not induce a significant change in the expression or nuclear localization of FLAG-IκBη. Taken together, these results indicate that IκBη is constitutively expressed in the nucleus regardless of the stimulation, and strongly suggest that IκBη is a nuclear IκB protein.

We constructed deletion mutants of IκBη to identify the domains required for the nuclear localization (Fig. S2*B*). FLAG-tagged full-length and deletion mutants of IκBη were expressed in NIH 3T3 cells and their distribution was examined by immunofluorescence staining (Fig. S2*C*). Full-length IκBη was present in the nucleus, but the mutants IκBη-ΔNA6 and IκBη-ΔCA2 lacking six ankyrin repeats at the N-terminal and two ankyrin repeats at the C-terminal, respectively, predominantly existed in the cytoplasm. The IκBη-ΔC mutant lacking the coiled-coil domain was present in both the nucleus and cytoplasm. These results indicate that ankyrin repeats are necessary for the nuclear localization, to which the C-terminal domain also contributes.

IκBη Interacts with the p50 Subunit of NF-κB. One key feature of IκB proteins is their interaction with NF-κB components, and nuclear IκB proteins were reported to interact with p50 or p52 rather than p65 (6). To test our hypothesis that IκBη is a unique nuclear IκB protein, we examined whether it interacts with NF-κB subunits. NIH 3T3 cells transiently expressing FLAG-IκBη were lysed and proteins immunoprecipitated with control, anti-p50, and anti-p65 antibodies were immunoblotted with anti-FLAG antibody (Fig. 3*A*). IκBη was coprecipitated with p50, but not with p65, indicating that IκBη is associated with a p50 homodimer or a heterodimer of p50 with an NF-κB subunit. Conversely, p50—but not p65—was coimmunoprecipitated with FLAG-tagged IκBη (Fig. 3*B*). We confirmed this interaction by using Myc-tagged NF-κB subunits (Fig. 3*C*). FLAG-IκBη was coimmunoprecipitated with Myc-p50, but not with Myc-p65. We also confirmed the interaction of endogenous proteins by using anti-IκBη antibody (Fig. S2*E*). These results indicate that IκBη interacts with NF-κB p50, rather than p65. It is known that ankyrin domains are important for the interaction with NF-κB (13). To determine which part is important for the association with p50, we carried out a coimmunoprecipitaton assay with IκBη deletion mutants (Fig. S2*D*). Full-length IκBη and the deletion mutant IκBη-ΔNA6 lacking the NH_2-teminal region of ankyrin repeats were expressed in NIH 3T3 cells, and the cell lysate was coimmunoprecipitated with anti-p50 antibody. Full-length IκBη, but not IκBη-ΔNA6, was found to associate with p50. These results indicated that ankyrin repeats are essential for the interaction with p50.

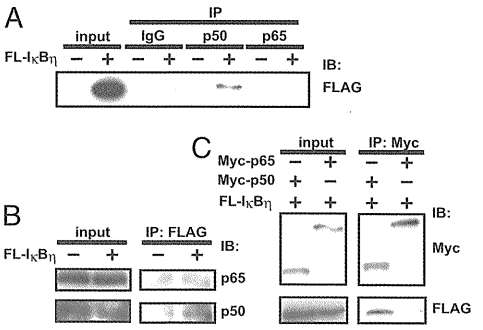

Fig. 3. Interaction of IκBη with the p50 subunit of NF-κB. (*A*) NIH 3T3 cells were transfected with control GFP or FLAG-tagged IκBη for 24 h and proteins were immunoprecipitated (IP) with anti-p50 Ab, anti-p65 Ab, or control rabbit IgG Ab. The immunoprecipitates were immunoblotted with anti-FLAG Ab. (*B*) NIH 3T3 cells were transfected as in *A* and proteins immunoprecipitated with anti-FLAG Ab. Whole-cell lysate or immunoprecipitated protein was immunoblotted with anti-p65 or anti-p50 Ab. (*C*) Cos7 cells were cotransfected with Myc-tagged p65 or p50 and FLAG-tagged IκBη for 24 h. Whole-cell lysates were immunoprecipitated with anti-Myc Ab, and immunoblotted with anti-FLAG Ab. Data shown are representative of two or three experiments.

IκBη Regulates the Expression of a Subset of Proinflammatory Genes in Innate Immune Responses. Nuclear IκB proteins are supposed to regulate the transcriptional activity of NF-κB either positively or negatively in the nucleus (7, 9, 14). To reveal the function of IκBη in innate immune responses, we first examined the effect of IκBη on transcription by NF-κB. A reporter plasmid containing three tandem repeats of the NF-κB binding site (3κB site) was transfected in NIH 3T3 cells with a control or IκBη expression vector. Expression of IκBη induced the activation of the reporter gene in NIH 3T3 cells in a dose-dependent manner (Fig. S3*A*). However, IκBη did not induce expression from the promoters in which the NF-κB binding sites were mutated. These results suggest that IκBη regulates the NF-κB signal transduction.

To confirm the function of IκBη as a regulator of NF-κB, we knocked down the expression of IκBη in Raw264.7 cells by using siRNA (Fig. 4*A*). After treatment with LPS, the production of TNF-α and IL-6 in the culture supernatant was measured by ELISA (Fig. 4 *B* and *C*). The proinflammatory cytokines were not expressed in unstimulated Raw264.7 cells and their expression was significantly up-regulated by LPS. Knockdown of IκBη in Raw264.7 cells suppressed the production of TNF-α and IL-6, although the magnitude of suppression differed. To further analyze the effects of IκBη knockdown on cytokine production in macrophages, the mRNA expression of *Tnfa*, *Il-6*, and *Ikbh* in Raw264.7 cells was quantitatively analyzed by real-time RT-PCR (Fig. S3 *B–D*). The level of *Ikbh* mRNA was reduced by IκBη siRNA regardless of LPS treatment. The expression of *Tnfa* and *Il-6* mRNA was induced by LPS stimulation, and was inhibited by IκBη siRNA. These results strongly suggest that IκBη regulates the NF-κB's transcriptional activity in the nucleus. To confirm these results, we further analyzed the mRNA expression of other genes induced at an early (*Cxcl1*, *Cxcl2*, *Il-1b*) or late (*Csf2*, *Csf3*) phase in response to LPS. Interestingly, although mRNA expression of *Cxcl2* (CXCL2) and *Il-1b* (IL-1β) was inhibited by IκBη siRNA, the expression of *Cxcl1* (CXCL1) was not significantly altered by IκBη siRNA (Fig.4 *D–F*). Similar to that of *Il-6*, the expression of *Csf2* (GM-CSF) and *Csf3* (G-CSF) mRNA was gradually induced by LPS in control cells, but was severely inhibited by IκBη siRNA (Fig.4 *G* and *H*). These results indicate that IκBη regulates the expression of a subset of NF-κB-mediated proinflammatory

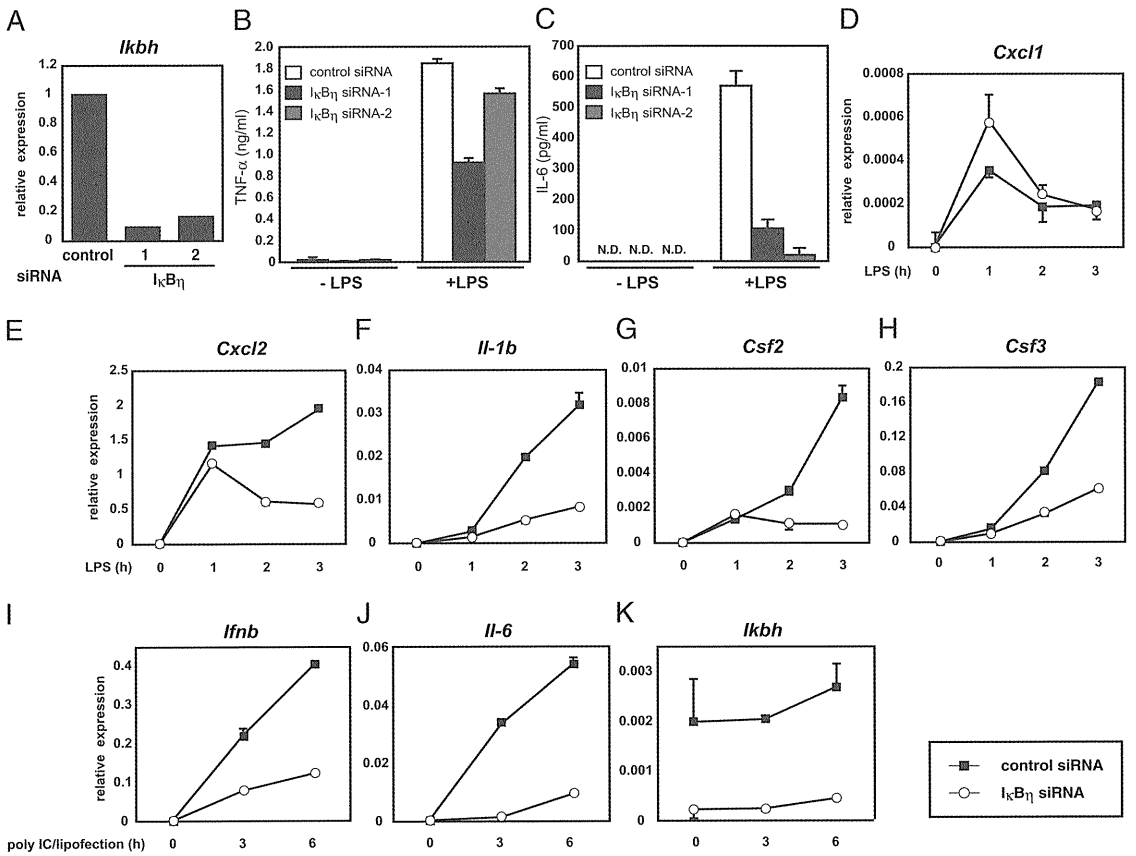

Fig. 4. IκBη regulates NF-κB-activated proinflammatory gene expressions in innate immune responses. (*A*) Raw264.7 cells were transfected with control or IκBη siRNA (1 or 2). Expression of IκBη mRNA was determined by real-time RT-PCR. The expression levels were normalized to that of the housekeeping gene *Hprt*. (*B* and *C*) Raw264.7 cells were transfected with control or IκBη siRNA, and treated with 100 ng/mL of LPS for 12 h. The levels of TNF (*B*) or IL-6 (*C*) in the culture supernatant were measured by ELISA. The data shown are representative of two experiments. (*D–H*) Raw264.7 cells were transfected with siRNA, and treated with 100 ng/mL of LPS. Total RNA was prepared at 0, 1, 2 and 3 h after the stimulation and quantitative RT-PCR was performed to measure mRNA levels of *Cxcl1* (*D*), *Cxcl2* (*E*), *Il-1b* (*F*), *Csf2* (*G*), *Csf3* (*H*). The expression levels were normalized to that of the housekeeping gene *Hprt*. Three independent experiments were performed and a representative set is shown. (*I–K*) Raw264.7 cells were transfected with control or IκBη siRNA for 26 h and then 0.5 μg/mL of poly (I:C) were transfected. Total RNA was prepared at 0, 3, and 6 h after the lipofection and mRNA expression levels of *Ifnb* (*I*), *Il-6* (*J*), and *Ikbh* (*K*) were measured by quantitative RT-PCR. The expressions were normalized to that of the housekeeping gene *Hprt*. The data shown are representative of three experiments.

cytokines. We also tested whether LPS-induced IL-10 expression affects the expression of inflammatory cytokines, because IL-10 is an anti-inflammatory cytokine induced to express by TLR stimuli and inhibits TLR-dependent gene expression (15, 16). A quantitative RT-PCR analysis revealed that IκBη knockdown did not enhance the LPS-induced *Il-10* mRNA expression in Raw264.7 cells, indicating that the suppression of LPS-induced production of proinflammatory cytokines and chemokines by IκBη siRNA was not due to the augmented expression of IL-10 (Fig. S3*E*).

It is also known that the cytosolic PRRs, such as RIG-I and MDA5, detect viral infection (17). Those RIG-I like receptors (RLRs)recognize cytosolic viral RNA and activate NF-κB to induce proinflammatory cytokines and IFNs (18). To further analyze the function of IκBη in innate immune responses, we examined the expression of *Ifnb* and *Il-6* mRNA in poly (I:C)-transfected Raw264.7 cells (Fig. 4 *I–K*). Although the expression of *Ifnb* and *Il-6* mRNA was gradually increased in poly (I:C)-transfected cells, the induction was significantly suppressed by IκBη siRNA. These results indicate that IκBη regulates the transcriptional activity of NF-κB not only in TLR signaling, but also in RLR signaling, and controls the transcription of a subset of proinflammatory cytokines in innate immune responses.

To further establish the function of IκBη, we knocked down expression of IκBη in another macrophage cell line, J774.1, and measured the production of proinflammatory genes (Fig. S3 *F–J*). IκBη siRNA significantly suppressed the expression of various proinflammatory cytokines, and the LPS-induced expression of *Il-6* mRNA in NIH 3T3 fibroblasts was also inhibited by IκBη siRNA (Fig. S3 *K* and *L*). These results indicate that IκBη plays a key role in regulating the expression of various NF-κB-mediated proinflammatory genes in various cells.

IκBη Regulates NF-κB Activity in the Nucleus. Nuclear IκB proteins are thought to modulate the activity of NF-κB at the transcriptional level, regulating the production of proinflammatory cytokines (6). To investigate the possible role of IκBη in the cytoplasmic signaling cascade, we first analyzed the effects of IκBη on LPS-induced degradation of IκBα by Western blotting (Fig. S4*A*). The IκBα level was reduced at 30 min after LPS

stimulation, regardless of the IκBη knockdown, indicating that IκBη has no effect on the stability of IκBα. We also analyzed LPS-induced phosphorylation of ERK1/2, p38, and JNK (Fig. S4*B*). Phosphorylation of these MAPKs was not altered by IκBη siRNA, indicating that knockdown of IκBη does not affect the LPS-mediated cytoplasmic signaling by MAPKs. Furthermore, we examined whether the nuclear translocation of NF-κB induced by stimuli was affected by IκBη siRNA (Fig. S4*C*). Nuclear localization of NF-κB was not affected by the knockdown of IκBη, indicating that IκBη does not regulate the nuclear translocation of NF-κB. Nuclear IκB proteins are supposed to interact with the target promoters to control transcriptional activity of NF-κB either positively or negatively (5). To address this possibility, we used an avidin-biotin-conjugated DNA-binding assay (Fig. S4*D*). Beads conjugated with the κB site of the IL-6 promoter sequence were added to lysate from Raw264.7 cells transfected with FLAG-IκBη, and the binding of p50 and p65 subunits to the IL-6 promoter sequence was analyzed by Western blotting. Although the IL-6 promoter fragment failed to pull-down p50 and p65 from unstimulated cells, both proteins were found to bind to the DNA fragment after LPS treatment. By contrast, FLAG-IκBη was found to bind to the DNA regardless of LPS stimulation. These results suggest that IκBη interacts with DNA to regulate the transcriptional activity of NF-κB.

Nuclear IκB proteins, BCL-3, IκBNS, and IκBζ, are rapidly induced by stimulation of TLRs and regulate NF-κB–mediated transcription. BCL-3 has been suggested to mainly control the transcription of primary response genes, and IκBNS and IκBζ regulate the expression of secondary response genes (10–12, 19). Because IκBη controls the expression of various proinflammatory genes, we examined the effect of IκBη siRNA on the LPS-induced expression of the three nuclear IκBs in Raw264.7 cells by real-time RT-PCR (Fig. S4 *E–G*). The siRNA did not alter the expression of these three IκBs, indicating that the inhibitory effect of IκBη siRNA on the expression of secondary response genes was not caused by the reduced expression of IκBNS and IκBζ. Similar results were also obtained by IκBη siRNA in J774.1 cells (Fig. S4*H*). Taken together, our results strongly suggest that IκBη directly regulates the expression of NF-κB-mediated transcription.

Discussion

NF-κB plays a central role in the inducible transcription of various proinflammatory genes. Prompt responses to inflammatory stimuli rely on repression of the transcriptional activity of NF-κB by inhibitors known as IκB proteins (1, 3). In the cytoplasm, canonical IκB proteins form a complex with NF-κB and inhibit its nuclear translocation (20). Extracellular and intracellular stimuli, such as TLR and RLR ligands, respectively, induce the ubiquitination and degradation of IκB, allowing NF-κB to move into the nucleus to regulate gene expression (17). In contrast to canonical IκB proteins, nuclear IκB proteins play a regulatory role in NF-κB–mediated transcription (6). In this article, we described a unique protein with eight ankyrin repeats, named IκBη, because of its structural similarity to the IκB family (Fig. 1*B*). We have also provided evidence that IκBη plays a crucial role as a nuclear IκB for regulating the NF-κB–mediated transcription of various proinflammatory genes in innate immune responses.

IκBη localizes in the nucleus and the subcellular localization is not affected by LPS treatment (Fig. 2 *B–D*). In addition, we found that IκBη binds to the p50 subunit of NF-κB via its ankyrin repeats (Fig. 3 *A–C*). Consistent with these results, it is known that nuclear IκB proteins prefer to interact with the p50 or p52 subunit of NF-κB, rather than other Rel family proteins (6). Although BCL-3, IκBNS, and IκBζ possess a nuclear localization signal motif and are predominantly located in the nucleus, IκBη lacks a canonical nuclear localization signal motif. Deletion analysis showed that the ankyrin repeats are absolutely necessary for the nuclear localization of IκBη, to which the coiled-coil domain also contributes (Fig. S2*C*). Moreover, compared with other nuclear IκB proteins whose expression is highly inducible through TLR signaling, IκBη is unusual because it is constitutively expressed in various tissues and only marginally up-regulated by TLR signaling (Fig. S2*A*).

Knockdown experiments using siRNA revealed that IκBη regulates NF-κB–mediated expression of a subset of proinflammatory genes in LPS-stimulated macrophages (Fig.4 *A–H* and Fig. S3 *D–J*). We also demonstrated that IκBη regulates the expression of *Ifnb* and *Il-6* mRNA in poly (I:C)-transfected macrophages (Fig. 4 *I–K*). These results strongly suggest that IκBη plays a crucial role for the expression of proinflammatory genes in innate immune responses. Moreover, IκBη positively regulates *Il-6* mRNA expression not only in macrophages but also in NIH 3T3 fibroblast cells, suggesting that IκBη regulates gene expression in various cell types (Fig. S3 *K* and *L*).

TLR-induced proinflammatory genes are divided into two subclasses, primary and secondary response genes (4). Primary response genes, such as *Tnfa* and *Cxcl2*, are expressed immediately in the absence of protein synthesis; the expression of secondary response genes, including *Il-6* and *Csf3*, requires newly synthesized mediators of NF-κB and occurs after the primary responses (21). IκBζ is considered to be essential for the induction of various secondary response genes, but not for primary genes, and IκBNS also regulates only secondary response genes (11, 22). In contrast to those known nuclear IκB proteins, IκBη regulates both types of genes, possibly because it is constitutively expressed in the nucleus (Fig. 4 *E–H* and Fig. S3 *B–J*).

As IκBη predominantly exists in the nucleus and it has no effect on the degradation of IκBα or nuclear translocation of NF-κBs, it is plausible that IκBη regulates NF-κB transcriptional activity in the nucleus (Fig. S4 *A–C*). In fact, we also showed that IκBη interacts with the DNA fragment of the IL-6 promoter, suggesting that IκBη regulates the transcriptional activity of NF-κB on promoters (Figs. S4*D* and S5). However, promoter activity of proinflammatory genes is strictly regulated in vivo by multiple mechanisms, such as chromatin remodeling, stable recruitment of transcriptional factors or cofactors, and posttranslational modification of NF-κB (12, 21, 23–25). Thus, it is possible that IκBη regulates chromatin remodeling as well as formation and stability of the transcriptional complex, and there may be an additional unknown mechanism for IκBη to modulate the transcriptional activity of NF-κB.

Although IκBη regulates the expression of a wide variety of proinflammatory genes, it has little or no effect on the expression of some NF-κB–regulated genes, such as *Cxcl1* and *Ikbz* (Fig. 4*D* and Fig. S4 *E–H*). It has been reported that NF-κB regulates expression of a distinct set of target genes by a distinct mechanisms, in cooperation with multiple regulatory factors, including p300/CBP cofactor or Trap80 subunit of the Mediator complex (24, 26). IκBη may also regulate a subset of NF-κB target genes on specific promoters or enhancers in a context-dependent manner. Taken together, the precise regulatory mechanism of IκBη still remains to be studied and is an interesting and important subject of further investigation.

In conclusion, IκBη is a unique nuclear IκB protein that contributes to NF-κB–mediated transcription, and plays an important regulatory role in innate immune responses by regulating the expression of proinflammatory cytokines (Fig. S5). Although this study focuses on the innate immune responses, ubiquitous expression of IκBη suggests that it may also play an important role for regulation of NF-κB signaling in other biological systems (27, 28).

Materials and Methods

Plasmids and Transfection. IκBη cDNA cloned by PCR was inserted into the vector pME-18S with a FLAG-tag, and p50 and p65 cDNA were cloned into

the vector pcDNA3.1 with a Myc-tag. The mutants IκBη-ΔNA6 (amino acids 251–516), IκBη-ΔCA2 (amino acids 1–230 plus 371–516) and IκBη-ΔC (amino acids 1–340) were constructed in pME-18S-FLAG. NIH 3T3 cells, Raw264.7 cells, and Cos7 cells were transfected using Attractene (Qiagen), according to the manufacturer's instructions.

RT-PCR and Real-Time RT-PCR. Raw264.7 cells, J774.1 cells, and NIH 3T3 cells were stimulated with 100 ng/mL of LPS or lipofected with 0.5 μg/mL of poly (I:C), and then total RNA was prepared using a High Pure RNA isolation kit (Roche Applied Science). The total RNA was reverse-transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Conventilnal PCR was performed with Blend Taq (TOYOBO). Quantitative real-time RT-PCR was performed on a LightCycler (Roche Applied Science) using SYBR Premix Ex Taq reagent (TaKaRa Bio Inc.). *Hprt* was used as an internal control. The primers used are listed in Tables S1 (RT-PCR) and S2 (real-time RT-PCR).

RNA Interference. For RNA interference experiments, synthetic siRNA (obtained as Stealth select RNA interference from Invitrogen) targeting mouse Ankrd42 (Table S3) or control siRNA in the mouse genome (Stealth RNAi-negative control, Medium GC duplex; Invitrogen) was transfected into Raw264.7 cells, J774.1 cells, or NIH 3T3 cells using HiPerFect (Qiagen) according to the manufacturers' directions. After 26 h of transfection, cells were collected and real-time PCR was conducted to examine the effect of the knockdown.

See *SI Materials and Methods* for further discussion.

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1. Hayden MS, Ghosh S (2008) Shared principles in NF-kappaB signaling. *Cell* 132: 344–362.
2. Li Q, Verma IM (2002) NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2:725–734.
3. Vallabhapurapu S, Karin M (2009) Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol* 27:693–733.
4. Akira S, Takeda K (2004) Toll-like receptor signalling. *Nat Rev Immunol* 4:499–511.
5. Ghosh S, Hayden MS (2008) New regulators of NF-kappaB in inflammation. *Nat Rev Immunol* 8:837–848.
6. Yamamoto M, Takeda K (2008) Role of nuclear IkappaB proteins in the regulation of host immune responses. *J Infect Chemother* 14:265–269.
7. Bours V, et al. (1993) The oncoprotein Bcl-3 directly transactivates through kappa B motifs via association with DNA-binding p50B homodimers. *Cell* 72:729–739.
8. Carmody RJ, Ruan Q, Palmer S, Hilliard B, Chen YH (2007) Negative regulation of toll-like receptor signaling by NF-kappaB p50 ubiquitination blockade. *Science* 317: 675–678.
9. Fiorini E, et al. (2002) Peptide-induced negative selection of thymocytes activates transcription of an NF-kappa B inhibitor. *Mol Cell* 9:637–648.
10. Kuwata H, et al. (2006) IkappaBNS inhibits induction of a subset of Toll-like receptor-dependent genes and limits inflammation. *Immunity* 24:41–51.
11. Yamamoto M, et al. (2004) Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. *Nature* 430:218–222.
12. Kayama H, et al. (2008) Class-specific regulation of pro-inflammatory genes by MyD88 pathways and IkappaBzeta. *J Biol Chem* 283:12468–12477.
13. Mosavi LK, Cammett TJ, Desrosiers DC, Peng ZY (2004) The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci* 13:1435–1448.
14. Yamazaki S, Muta T, Takeshige K (2001) A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. *J Biol Chem* 276:27657–27662.
15. Cao S, Zhang X, Edwards JP, Mosser DM (2006) NF-kappaB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. *J Biol Chem* 281:26041–26050.
16. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683–765.
17. Takeuchi O, Akira S (2010) Pattern recognition receptors and inflammation. *Cell* 140: 805–820.
18. Kato H, et al. (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441:101–105.
19. Kuwata H, et al. (2003) IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-alpha production in macrophages. *Blood* 102:4123–4129.
20. Savinova OV, Hoffmann A, Ghosh G (2009) The Nfkb1 and Nfkb2 proteins p105 and p100 function as the core of high-molecular-weight heterogeneous complexes. *Mol Cell* 34:591–602.
21. Ramirez-Carrozzi VR, et al. (2006) Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. *Genes Dev* 20:282–296.
22. Hirotani T, et al. (2005) The nuclear IkappaB protein IkappaBNS selectively inhibits lipopolysaccharide-induced IL-6 production in macrophages of the colonic lamina propria. *J Immunol* 174:3650–3657.
23. Leung TH, Hoffmann A, Baltimore D (2004) One nucleotide in a kappaB site can determine cofactor specificity for NF-kappaB dimers. *Cell* 118:453–464.
24. Dong J, Jimi E, Zhong H, Hayden MS, Ghosh S (2008) Repression of gene expression by unphosphorylated NF-kappaB p65 through epigenetic mechanisms. *Genes Dev* 22: 1159–1173.
25. Chen LF, et al. (2005) NF-kappaB RelA phosphorylation regulates RelA acetylation. *Mol Cell Biol* 25:7966–7975.
26. van Essen D, Engist B, Natoli G, Saccani S (2009) Two modes of transcriptional activation at native promoters by NF-kappaB p65. *PLoS Biol* 7:e73.
27. Pasparakis M (2009) Regulation of tissue homeostasis by NF-kappaB signalling: Implications for inflammatory diseases. *Nat Rev Immunol* 9:778–788.
28. Broide DH, et al. (2005) Allergen-induced peribronchial fibrosis and mucus production mediated by IkappaB kinase beta-dependent genes in airway epithelium. *Proc Natl Acad Sci USA* 102:17723–17728.

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Dlk-1, a cell surface antigen on foetal hepatic stem/progenitor cells, is expressed in hepatocellular, colon, pancreas and breast carcinomas at a high frequency

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Delta-like 1 protein (Dlk-1), also known as preadipocyte factor 1 (Pref-1), is a transmembrane and secreted protein with epidermal growth factor (EGF)-like repeats. Dlk-1 is known to be expressed in foetal liver, but absent in neonatal and adult liver in mice and rats. Dlk-1 is also expressed in a subpopulation of hepatic oval cells, which are considered as stem/progenitor cells in rat adult liver. In this study, we generated monoclonal antibodies against human Dlk-1 (hDlk-1) and investigated hDlk-1 expression in human liver and hepatocellular carcinoma (HCC). Like rodent livers, hDlk-1 was detected in foetal liver, but not in adult liver. In HCC, hDlk-1 was positive for 20.5% of the cases examined and was localized in both cytoplasm and cell membrane, whereas hDlk-1 was undetected in viral hepatitis, nodular cirrhosis. Interestingly, hDlk-1 positive HCC was found more frequently in younger patients and its expression was correlated with alpha-fetoprotein expression. Furthermore, hDlk-1 was also detected frequently in colon adenocarcinomas (58%), pancreatic islet carcinoma (50%), and small cell lung carcinoma (50%). Thus, hDlk-1 is a cell surface protein expressed in many carcinomas including HCC and may be a potential target for monoclonal antibody therapy for carcinomas.

Keywords: cell surface protein/colon adenocarcinoma/hDlk-1/hepatocellular carcinoma (HCC)/small cell lung carcinoma.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence activated cell sorter; His, histidine; MEM, minimum essential medium; mRNA, messenger RNA; PBS, phosphate-buffered saline.

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours in the world (1). While the occurrence has been unusually high in Asia and Africa, it is recently increasing in United States and the incidence and mortality rates are anticipated to double over the next 10• 20 years (2, 3). HCC is often diagnosed at an advanced stage when curative therapies are of limited efficacy. In order to reduce morbidity and mortality of HCC, it is of prime importance to develop a system for early diagnosis, novel systemic therapies for the advanced disease as well as means to prevent HCC development.

Dlk-1 protein, also known as Pref-1, foetal antigen 1 (FA1), pG2 and ZOG, is a transmembrane and secreted protein, which is a member of the epidermal growth factor (EGF)-like family including Notch/Delta/Serrate (4• 8). Dlk-1 is strongly expressed in foetal tissues such as liver, pancreas and skeletal muscle, but its expression is restricted in adult tissues such as placenta and adrenal grand (9• 12). As there are many receptors and ligands in the EGF-like family proteins, which regulates cell fate and differentiation during development in many organisms, Dlk-1 may also play a role in development and differentiation (12• 14). In fact, there are several reports showing the involvement of Dlk-1 in adipogenesis (15), hematopoiesis (16, 17) and development of pancreas (18, 19), placenta (20) and adrenal gland (21, 22). In addition to the normal tissues, Dlk-1 was also shown to be expressed in several tumours, such as neuroblastoma (23), glioma (24), small cell lung carcinoma (25), myelodysplastic syndrome, acute myelogenous leukaemia (26), etc. (27, 28). These results suggest that Dlk-1 may play an important role in tumourigenesis as well as organogenesis.

Previously, we demonstrated that Dlk-1 is strongly expressed on the cell surface of hepatoblasts in murine foetal liver from embryonic day (ED) 10.5• 16.5 and Dlk-1• cells isolated from foetal liver showed high-proliferative activity and bi-potentiality (10). Its expression is down-regulated in late gestation and completely absent after birth. In liver injury under conditions that limit proliferation of hepatocytes, immature cells with oval shaped nucleus called hepatic oval cells appear around the portal vein. As they are proliferative and express markers of hepatocytes and cholangiocytes, hepatic oval cells have been considered as adult liver progenitors (29). Similar cells were also shown to be present in severe hepatitis and implicated in tumourigenesis (30). The expression of Dlk-1 was also observed in a subpopulation of rat oval cells induced by the 2-acetylaminofluorene/partial hepatectomy model (31). These data suggest that Dlk-1 is a cell

surface antigen of foetal/adult hepatic stem/progenitor cells.

It has become clear that tumour, in many cases, is a heterogeneous cell population and only a small fraction of the cells possess the potential to self-renew. Cancer stem cell or tumour initiating cell, which was first documented in haematological malignancies, has subsequently been discovered in many solid tumours, including breast, brain, prostate, liver, lung, melanoma, pancreas and colon tumours (32• 36). While it was shown that CD133, known as a stem cell marker, is expressed in cancer stem cells in many tumours including HCC (37• 41), the relation between normal tissue stem cells and cancer stem cells is not clear in most of the cases.

In this study, we established many hybridoma clones which produced anti-hDlk-1 monoclonal antibodies (mAb). Among them, we selected three independent clones usable for immunohistochemistry and characterized these antibodies by flow cytometry. Using these mAbs which recognized a different epitope, we investigated the expression of human Dlk-1 (hDlk-1) during liver development by immunohistochemistry. The expression of hDlk-1 showed a pattern similar to mouse Dlk-1 during liver development, suggesting that hDlk-1 is also a marker of hepatic stem/progenitor cells in embryo. We then examined hDlk-1 expression in human neoplastic liver lesions. About a half of HCC specimens from under 40-years-old patients expressed hDlk-1, whereas the positive ratio of hDlk-1 over 50-years-old patients was • 10%. Our study indicates the possibility that hDlk-1 is a common cell surface antigen both in human foetal liver stem/progenitor cells and in a part of HCC. Moreover, hDlk-1 was also frequently expressed in colon, breast, pancreas and lung carcinoma. These observations suggest that hDlk-1 is a potential target for monoclonal antibody-based therapy in those carcinomas.

Materials and Methods

Plasmid constructs

Full length hDlk-1 and its derivatives (EGF1-3 and EGF 4-6) were amplified by PCR. The sequences of primers were as follows: Fw1: 5'-cgcgtccgcaaccagaagccc-3', Rv1: 5'-aagcttgatctcctcgtcgccggcc-3' (for full length hDlk-1), Fw2: 5'-gcggccgcgctgaatgcttcccggcc-3', Rv2: tctagaggcccgaacatctctatcac-3' (for hDlk-1 EGF1-3), Fw3: 5'-gcggccgcgcctgctcctcggcccccc-3', Rv3: 5'-gcgtatagtaagctctgcgg-3' (for hDlk-1 EGF4-6). All PCR products were verified by DNA sequencing. Full-length hDlk-1 cDNA was cloned in pcDNA3 vector (Invitrogen, Carlsbad, CA) with Flag tag. hDlk-1 EGF1-3 cDNA was subcloned in pME18SNeo carrying the signal sequence of CD8, His tag, and transmenbrane and cytoplasmic domains of FXYD5, which was kindly gifted by Dr Tanaka, M. (University of Tokyo, Tokyo, Japan). hDlk-1 EGF4-6 cDNA was subcloned in pME18SNeo containing the signal sequence of CD8, His tag.

Antibodies

Mouse monoclonal antibodies against hDlk-1 (clone DI-6, DI-2-20 and DI-4-22) were generated by the DNA immunization method (Nosan Corp., Kanagawa, Japan). To prepare purified monoclonal antibodies, hybridoma clones (3• 10^6 cells) were intraperitoneally administered to BALB/c Slc-nu/nu mice (Japan SLC, Shizuoka, Japan), which received 2,6,10,14-tetramethylpentadecane (Sigma Aldrich Japan K.K., Tokyo, Japan) 7 days before injection of hybridoma. After collection of ascites, the antibodies were purified with a protein G column (GE Healthcare, Buckinghamshire, England).

Rabbit polyclonal antibodies against hDlk-1 were prepared by immunizing with peptides containing the extracellular domain of hDlk-1 except for the putative signal sequence. Polyclonal antibodies were purified by affinity chromatography using columns conjugated with the peptides used for immunization.

Cell culture, transfection and flow cytometry

COS7 cells, HEK-293 cells, Huh-7 cells and SK-N-FI cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum. HepG2 cells and C3A/HepG2 cells were maintained in MEM supplemented with 10% foetal bovine serum. COS7, HEK-293, Huh-7 and HepG2 cells were from Human Science Research Resource Bank (Osaka, Japan). SK-N-FI and C3A/HepG2 cells were purchased from American Type Culture Collection (Rockville, MD). Transfection was performed using Lipofectoamine and Plus reagent (Invitrogen). To establish HEK-293 cells stably expressing hDlk-1 (293-hDlk-1), HEK-293 cells were transfected with pcDNA3 vector containing full-length hDlk-1 cDNA and selected with G418 (Invitrogen). COS7 cells were transiently transfected with expression constructs containing either hDlk-1 EGF1-3 or hDlk EGF4-6, and two days after transfection, these cells were harvested and subjected to fluorescence activated cell sorter (FACS) analysis. 293-hDlk-1 cells were cultured to subconfluency and harvested for flow cytometry by FACSCalibur (Nippon Becton Dickinson, Tokyo, Japan).

Immunohistochemistry

Tissue arrays and sections of tumours used in this study were purchased from Cybrdi (Rockville, MD), Shanghai Outdo Biotech Co. (Shanghai, China), Super Bio Chips (Seoul, Korea), ISU ABXIS (Seoul, Korea), US Biomax (Rockville, MD). Clinical information of patients (age, sex, grade and pathology diagnosis) is described in their homepage and data sheets. Foetal liver specimens were purchased from Biochain (Hayward, CA).

Paraffin embedded tissue sections and arrays were deparaffinized, and then autoclaved for 5 min in citrate buffer (pH 6.0) or TE buffer (pH 9.0). Slides were treated with methanol containing 0.3% H_2O_2 to inhibit endogenous peroxidase activity, and incubated with anti-hDlk-1 mAbs (10 mg/ml) at 4°C over night. After washing with phosphate-buffered saline (PBS), sections were stained with Vectastain ABC Elite kit (Vector, Burlingame, CA) and then counterstained with haematoxylin (Wako, Osaka, Japan). HCC sections with more than 10% immunopositive cells, either cell membrane or cytoplasmic stainin, were considered as positive.

Result

Characterization of anti-hDlk1 monoclonal antibodies

We established over 100 hybridoma clones producing anti-hDlk-1 mAb. Among them, three independent clones usable for immunohistochemistry in paraffin-embedded tissue sections were selected. First, we evaluated the reactivity and specificity of these antibodies by flow cytometry. Three mAbs against hDlk-1, DI-6, DI-2-20 and DI-4-22, specifically recognized HEK-293 cells stably expressing hDlk-1 (Fig. 1B), but not parent HEK-293 cells (not shown). On the other hand, these antibodies failed to recognize mouse Dlk-1, which shares • 90% similarity with hDlk-1 at the amino acid level (data not shown). These results confirmed that anti-hDlk-1 mAbs, DI-6, DI-2-20 and DI-4-22, specifically recognize hDlk-1. We then mapped the region of hDlk-1 to which the antibodies bind using deletion mutants of hDlk-1 (Fig. 1A). As shown in Fig. 1D, DI-6 recognized the EGF repeats 1• 3 (amino acid 24• 129), whereas DI-2-20 and DI-4-22 recognized EGF repeats 4• 6 (amino acid 126• 382). DI-2-20 and DI-4-22 recognized the same epitope because they competed each other (results not shown).

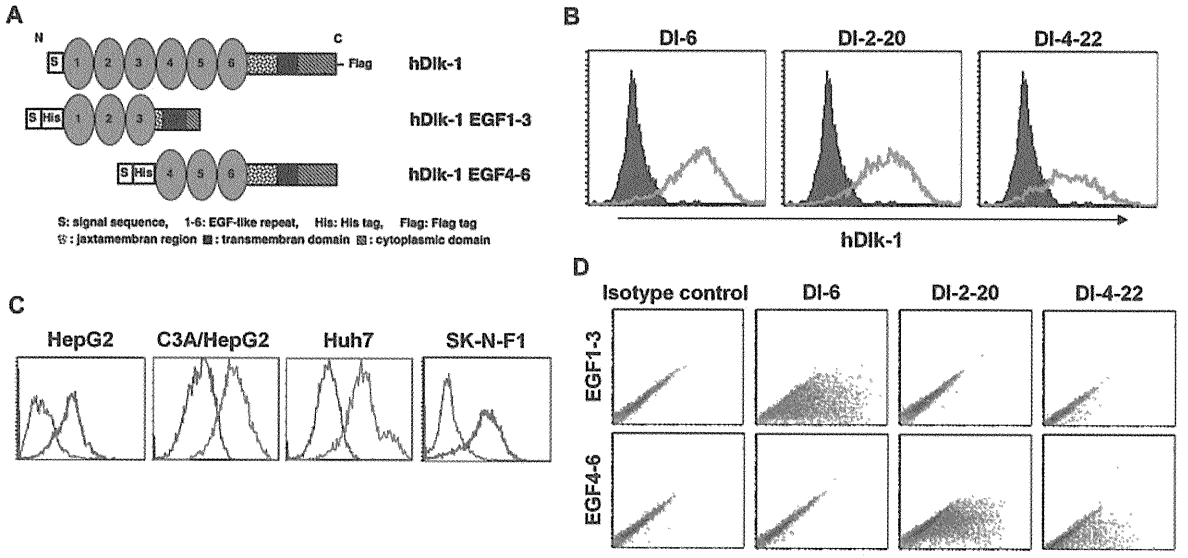

Fig. 1 Characterization of anti-hDlk-1 monoclonal antibodies by FACS analysis. (A) Schematic representation of various hDlk-1 constructs used in this study. (B) Three monoclonal antibodies used in this study specifically recognize hDlk-1 expressing cells. HEK-293 cells stably-expressing hDlk-1 were harvested, dispersed in a single cell suspension, and analysed by flow cytometry using monoclonal antibodies against hDlk-1, DI-6, DI-2-20, DI-4-22 (green line). Blue area: isotype control (mouse IgG1). (C) Anti-hDlk-1 mAb DI-2-20 also recognizes endogenous hDlk-1 in human cancer cell lines. Cancer cell lines indicated here were harvested and subjected to FACS analysis by using DI-2-20, respectively. Blue line: isotype control (mouse IgG1), red line: anti-hDlk-1 mAb (DI-2-20). Another monoclonal antibody, DI-6, showed similar result (data not shown). (D) Identification of the region of hDlk-1 to which anti-hDlk-1 mAbs bind. COS7 cells were transiently transfected with plasmids containing a various domain of hDlk-1 represented in (A). Two days after transfection, these cells were harvested and subjected to FACS analysis with anti-hDlk-1 mAb, DI-6, DI-2-20 and DI-4-22, respectively. DI-2-20 and DI-4-22 recognize EGF repeat 4• 6, whereas DI-6 recognizes EGF repeat 1• 3.

As reported previously, hDlk-1 was expressed early in liver development, but not in adult liver (9). To confirm that selected mAbs were suitable for immunohistochemistry in paraffin-embedded tissue sections, the expression pattern of hDlk-1 protein in liver was examined by immunohistochemical staining using DI-2-20 mAb (Fig. 2). A foetal liver tissue at 22 weeks (22w) showed membrane and cytoplasmic staining of hDlk-1 in hepatocytes. However, hDlk-1 was not detected in foetal liver at 38 weeks (38w) and adult liver. Northern blot analysis showed that hDlk-1 mRNA was strongly expressed in foetal liver from 6 to 12 weeks of gestation (not shown). The expression of hDlk-1 showed a pattern similar to mouse Dlk-1 during liver development, suggesting that hDlk-1 is also a marker of hepatic stem/progenitor cells in embryo. Similar results were obtained with either DI-6 or DI-4-22 mAb, though less sensitive than DI-2-20 (not shown). These results suggest that DI-2-20 was also useful for detecting endogenous hDlk-1 by immunohistochemistry. Therefore, we used mainly DI-2-20 mAb for further analysis.

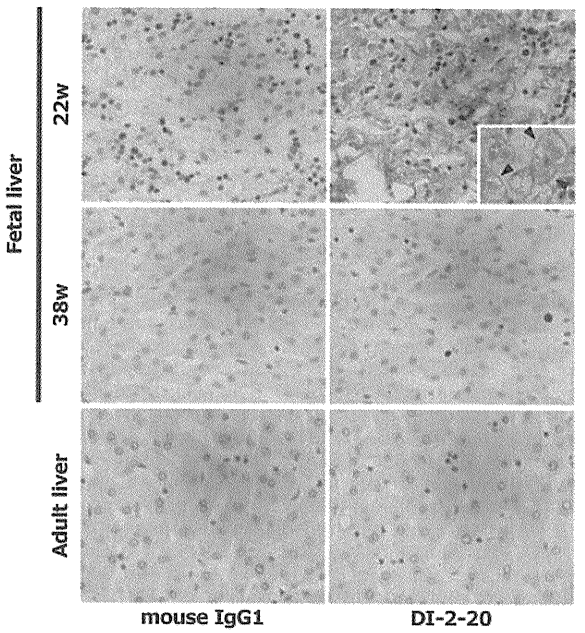

Fig. 2 Immunohistochemical analysis of hDlk-1 in foetal and adult liver. Each specimen was stained with anti-hDlk-1 mAb DI-2-20 (right panels). Foetal liver tissue at 22w (upper) shows membrane (arrow heads) and cytoplasmic staining with DI-2-20 monoclonal antibody. hDlk-1 staining was not observed in foetal liver at 38w (middle) and adult liver (lower). Mouse IgG1 used as isotype control was negative (left panels). Magnification is • 400.

Expression of hDlk-1 in HCC cells

We then examined the cell surface expression of hDlk-1 in a number of cancer cell lines originated from HCC by flow cytometry by using DI-6 and DI-2-20 antibodies. Among them, significant cell surface expression of hDlk-1 was detected in HepG2, C3A/HepG2 and Huh-7 cells (Fig. 1C). These results suggest that hDlk-1 is the cell surface antigen of HCC

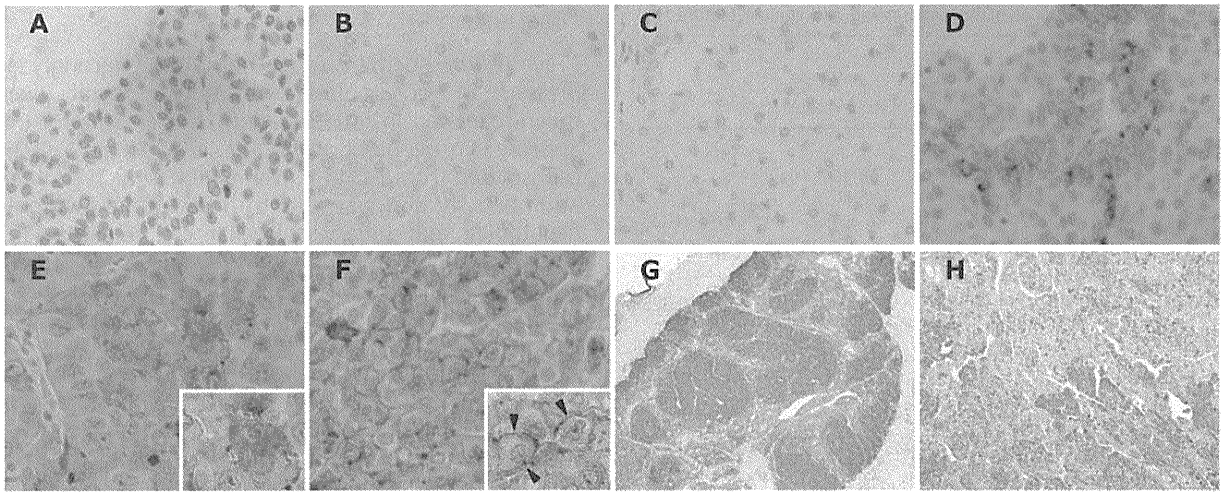

Fig. 3 hDlk-1 expression was observed only in hepatocellular carcinoma. Immunohistochemical staining with anti-hDlk-1 monoclonal antibody DI-2-20 was performed using tissue microarrays of liver tumour (388 malignant and 19 benign), nodular cirrhosis (40), viral hepatitis (11) and normal liver (26). The expression of hDlk-1 was detected only in HCC sections (D• F), not in adult liver (not shown), viral hepatitis (B), nodular cirrhosis (C), cavernous hemangioma (not shown) and intrahepatic cholangioma (not shown). hDlk-1 immunoreactivity in HCC cells was localized in either cytoplasm (E, inset) or cell membrane (F, inset). Membranous staining of hDlk-1 was shown by arrow heads. hDlk-1 expression was also examined in some samples of conventional tissue section corresponding to hDlk-1 positive spots in tissue array (G and H). Mouse IgG1 was used as negative control (A). Magnification is • 400 (A• F), • 1000 (inset in E and F) and • 40 (G and H).

cells. Additionally, hDlk-1 was also expressed in the cell surface of SK-N-F1 cells, a neuroblastoma cell line (Fig. 1C).

To evaluate the expression of hDlk-1 in non-neoplastic and neoplastic liver lesions, immunohistochemical analysis was performed by using tissue arrays. Typical staining profiles are shown in Fig. 3 and the results are summarized in Table I. hDlk-1 expression was undetectable at all in normal adult liver (0/26, not shown), non-neoplastic liver lesions, viral hepatitis (0/11, Fig. 3B) and nodular cirrhosis (0/40, Fig. 3C). hDlk-1 was also not found in cavernous hemangioma (0/19, not shown) and intrahepatic cholangioma (0/2, not shown). In contrast, hDlk-1 expression examined by DI-2-20 antibody was specifically observed in HCC and was positive for 79 out of 386 cases (20.5%, Fig. 3D• F). The pattern of hDlk-1 staining varied among individual tumours, e.g. hDlk-1 signal exhibited a uniform distribution within the tumour in one case (Fig. 3E, F), whereas it showed mosaic-like pattern in another case (Fig. 3D). Similar results were obtained with the same tissue array by using DI-6 antibody that recognizes a different epitope from DI-2-20 (not shown). Although hDlk-1 is a type I transmembrane protein, the immunoreactivity in HCC cells was mainly observed in the cytoplasm (Fig. 3E, inset), whereas hDlk-1 was expressed in cell surface in some cases (Fig. 3F, inset). We also examined some of conventional tissue slides corresponding to hDlk-1 positive spots in tissue arrays for hDlk-1 expression. The staining of hDlk-1 was not uniform, but covered more than 10% of the tumour in all tissue sections that we studied (Fig. 3G and H). No immunoreactivity was observed in normal tissues adjacent to the tumour (not shown).

Table I. Summary of immunohistochemical analysis.

	hDlk-1 staining	
	•	•
(A) hDlk-1 expression in HCC (386 cases)		
CS03-01-002 (Cybrdi)	40	15
CC03-01-001 (Cybrdi)	43	12
CC03-01-003 (Cybrdi)	46	10
CC03-02-001 (Cybrdi)	14	3
A204 (ISU ABXIS)	29	6
A204(II) (ISU ABXIS)	29	6
BC03013 (Biomax US)	49	10
OD-CT-DgLiv02-002 (Outdo bio.)	25	7
CS3 (HCC only) (Super Biochips)	32	10
Total	307 (79.5%)	79 (20.5%)
(B) hDlk-1 expression in normal liver, non-neoplastic liver lesions, benign liver tumour and cholangiocarcinoma		
Normal liver	23	0
Viral hepatitis	11	0
Nodular cirrhosis of liver	40	0
Cavernous hemangioma of liver	19	0
Intrahepatic cholangicarcinoma	2	0

As summarized in Table II, there was no clear correlation between hDlk-1 expression and pathological grade, gender, or aetiology such as HBV or HCV infection ($P > 0.05$ by $\cdot^2$ test). In contrast, hDlk-1 expression was clearly correlated with age or expression of alpha-fetoprotein (AFP). Interestingly, hDlk-1 expression was detected at higher frequency in HCC under 50 years old (51 out of 162 specimens, 31.5%), whereas the hDlk-1 positive HCC was dramatically decreased over 50 years old (28 out of 220 specimens, 12.7%). Especially, the hDlk-1 positive HCC was 43.1% (22 out of 51 specimens) under 40 years old. AFP is a well-established marker for HCC and was

Table II. Relationship of hDlk-1 expression and clinical features.

	hDlk-1			
	•	•	hDlk-1• ratio	
Grade				
I	46	9	16.40%	
II	158	54	25.50%	
III	50	14	21.90%	P• 0.347
Gender				
Male	245	59	19.40%	
Female	58	20	25.60%	P• 0.225
Age				
404	29	22	43.10%	
40• 49	82	29	26.10%	
50• 60	99	17	14.70%	
460	93	11	10.60%	P50.01
AFP				
•	179	25	12.30%	
•	26	25	49.00%	P50.01
Aetiology				
HBV•	44	12	21.40%	
HCV•	6	0	0.00%	
•	7	1	12.50%	P• 0.393

present in 51 of 255 cases (20.0%). hDlk-1 was detected in 25 of 51 AFP-positive HCCs (49.0%), whereas 25 of 204 AFP-negative HCCs (12.3%) were positive for hDlk-1. These results indicated that the hDlk-1 was expressed more frequently in a patient under 50 years old and in AFP-positive HCC.

Expression of hDlk-1 in other carcinomas

We then examined the expression of hDlk-1 in various carcinomas and found that hDlk-1 was highly and frequently expressed in colon adenocarcinoma (58.6%), breast carcinoma (39.0%), pancreatic carcinoma (30.8%) and lung carcinoma (30.2%), but not in ovarian carcinoma (13.2%) and gastric carcinoma (3.33%). Interestingly, in pancreatic carcinomas, hDlk-1• cells were found more frequently in islet carcinoma (50.0%) than duct carcinoma (28.3%). In the lung carcinoma, hDlk-1 was expressed in small cell lung carcinoma (52.5%), but only few non-small cell carcinoma (8.9%) expressed hDlk-1 (Fig. 4, summarized in Table III). These results suggested that hDlk-1 was expressed in various carcinomas.

As described above, hDlk-1 was more frequently expressed in AFP-positive HCC. Therefore we examined the expression of hDlk-1 in other AFP positive cancer, AFP-producing gastric cancer. hDlk-1 was rarely expressed in gastric carcinoma (3.33%), but was positive for two out of 10 cases in AFP-producing gastric cancer (20.0%, Fig. 4 G and H, summarized in Table III). Previously, Dezso et al. (42) reported that hDlk-1 was highly expressed in hepatoblastoma, AFP-positive liver cancer occurring in childhood. Together with our result, it was suggested that hDlk-1 was frequently expressed in AFP-positive cancers.

Discussion

Previously, we demonstrated that Dlk-1 is strongly expressed in hepatoblasts in mouse foetal liver, down-regulated in late gestation, and completely disappeared in neonatal and adult liver. Single Dlk-1• cell isolated from ED14.5 liver exhibited high proliferating activity and was able to differentiate into both hepatocyte and biliary epithelial cell lineages (10). These findings suggested that Dlk-1 is a cell surface antigen of foetal hepatic stem/progenitor cells in the mouse. In this study we prepared mAbs against hDlk-1 and showed that the expression pattern of hDlk-1 is similar to mouse Dlk-1/Pref-1 during liver development, i.e. hDlk-1 is expressed in foetal liver but not in adult liver and hDlk-1 is present in both cell membrane and cytoplasm (Fig. 2). Thus, hDlk-1 may be an excellent marker of foetal hepatic stem/progenitor cells in human as well.

In adult liver, hepatic progenitor cells (HPCs) appear around the portal vein when liver is severely injured. These cells are known as hepatic oval cells in rodents and express markers of both hepatocytes and biliary epithelium (29). HPCs in chronic liver diseases are suggested to contribute to liver regeneration as well as hepatocarcinogenesis (43• 45). Dlk-1 is not expressed in normal liver and was found in a subpopulation of hepatic oval cells induced in rats treated with 2-acetylaminofluorene and partial hepatectomy, a well-established rat model of hepatic oval cell induction (31). However, in a mouse model of hepatic oval cell induction by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, Dlk-1 is not expressed in hepatic oval cells (46). In the present study, hDlk-1 immunoreactive cells were not found in the specimens of viral hepatitis and nodular cirrhosis. In contrast, it was expressed frequently in HCC, but not in intrahepatic cholangioma, cavernous hemangioma and non-neoplastic liver lesions. These results demonstrate that hDlk-1 is expressed in HCC at high frequency, but do not exclude the possibility that Dlk-1 is expressed in some of adult hepatic stem/progenitor cells in chronically injured liver, which can lead to tumourigenesis.

It still remains unclear whether liver tumour is derived from hepatic stem/progenitor cells or mature hepatocytes. Recently, Lee et al. (47) reported that two subtypes (HB and HC) of HCC were categorized by analysis of gene expression patterns, and suggested that they may reflect the origin of tumour cells. The HB subtype shared a gene expression pattern with foetal hepatoblasts, whereas the HC subtype shared with adult hepatocytes, suggesting that the HB subtype may arise from hepatic stem/progenitor cells. The HB subtype of HCC showed poor prognosis compared to HC subtype. Interestingly, the HB subtype accounts for • 20% of HCC examined, similar to the frequency of hDlk-1 positive cells. In addition, the expression of hDlk-1 was more frequently found in HCC patients younger than 50 years old. Because HCC develops after a long latency period of chronic infection with HBV, HCV or both, the incidence of HCC is relatively high over 50 years old. Therefore, our finding that HCC patients under 50 years old expressed hDlk-1

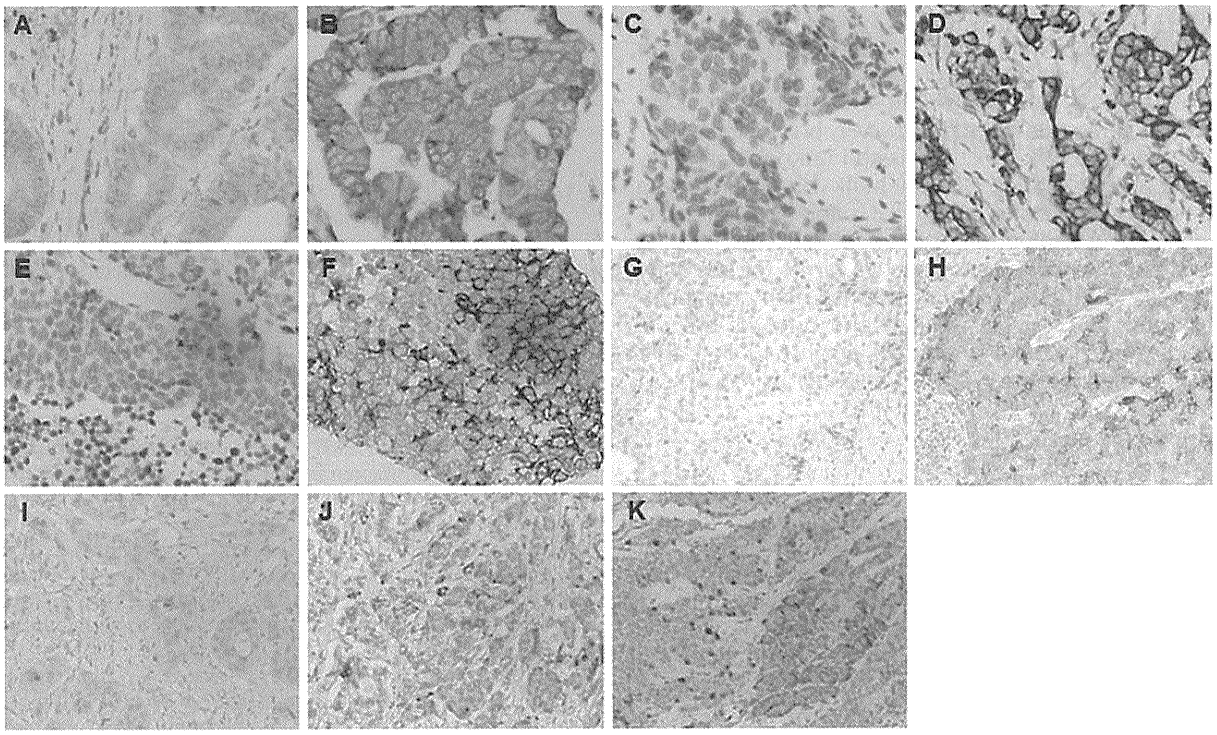

Fig. 4 hDlk-1 expression in various tumours. hDlk-1 expression in various tumours were examined by immunohistochemistry using anti-hDlk-1 mAb, DI-2-20. (A and B) Colon adenocarcinoma, (C and D) breast carcinoma, (E and F) small cell lung carcinoma, (G and H) AFP-producing gastric cancer, (I and J) pancreatic adenocarcinoma, (K) islet cell carcinoma. Tumour cells showed strong staining in cytoplasm (J and K), and cytoplasm and cell membrane (B, D, F and H). A, C, E, G and I showed hDlk-1 negative tumour cells. Magnification is • 200.

Table III. Summary of hDlk-1 staining in various tumours.

Tumour		Dlk•	Dlk•	Total
Colon adenocarcinoma		24 (41.4%)	34 (58.6%)	58
Ovarian carcinoma		59 (86.8%)	9 (13.2%)	68
Pancreatic carcinoma	Duct adenocarcinoma	33 (71.7%)	13 (28.3%)	46
	Islet cell carcinoma	3 (50.0%)	3 (50.0%)	6
Breast carcinoma		36 (61.0%)	23 (39.0%)	59
Lung carcinoma	NSCLC	51 (91.1%)	5 (8.9%)	56
	SCLC	19 (47.5%)	21 (52.5%)	30
Gastric carcinoma		29 (96.7%)	1 (3.33%)	30
AFP-producing gastric cancer	8 (80%)	2 (20%)	10	

more frequently is unexpected and intriguing. However, there was so far no clear correlation between hDlk-1 positive tumours in patients under 50 years old and specific aetiologies such as gender, pathological grade and stage. Recently, Huang et al. (48) also reported that hDlk-1 expression in HCC showed no significant correlation with HBV infection, tumour size and serology of AFP. Thus, our finding suggests that hDlk-1• HCC develops in a relatively short latency period and may have an origin different from other HCC with a longer latency period. Alternatively, considering the recent finding that albumin positive hepatocytes can be converted into induced pluripotent stem cells (iPS) by transient expression of c-Myc, Sox2, Oct3/4 and Klf4 (49), conversion of mature hepatocytes to an immature stage with hDlk-1 expression may occur during chronic liver injury. Thus, it is tempting to speculate that hDlk-1 may be a hallmark of HCC originated from hepatic or cancer stem/progenitor cells. The origin and mechanism of tumourigenesis of HCC still need extensive investigation.

While Dlk-1, also known as Pref-1, was originally described as an inhibitor of adipogenesis (8), the precise function still remains unknown. In this study, we showed that hDlk-1 is expressed in not only HCC but also many carcinomas such as colon, breast, pancreatic and lung carcinomas. As previously reported, colony formation, cell growth and tumourigenicity of HCC cell lines were significantly decreased when the endogenous hDlk-1 was knocked down by RNAi (48), and hDlk-1 promoted proliferation of glioblastoma cell line (GBM cells) (24) and erythroid leukemia cell line (K562 cells) (26). Furthermore, Dlk-1 has been reported to interact with Notch 1, and modulate

Notch signalling as a negative regulator (50). Notch 1 signalling prevented HCC cells to proliferate by induction of cell cycle arrest and apoptosis (51). Thus, hDlk-1 may contribute to tumourigenesis by enhancing tumour growth. However, precise molecular mechanism of Dlk functions is still unknown, and requires further studies. Because hDlk-1 is a cell surface molecule expressed in many HCCs and also other carcinomas, but neither in normal adult liver nor most of the tissues, it may be an attractive target for antibody therapy. In this study, we established many monoclonal antibodies against hDlk-1, and now we are developing monoclonal antibodies against hDlk-1 that block proliferation of HCC in a xenograft model.

Conflict of interest
None declared.

References

1. Parkin, D.M., Bray, F., Ferlay, J., and Pisani, P. (2001) Estimating the world cancer burden: Globocan 2000. Int. J. Cancer 94, 153• 156
2. El-Serag, H.B. and Mason, A.C. (1999) Rising incidence of hepatocellular carcinoma in the United States. New Engl. J. Med. 340, 745• 750
3. Okuda, K. (2000) Hepatocellular carcinoma. J. Hepatol 32, 225• 237
4. Halder, S.K., Takemori, H., Hatano, O., Nonaka, Y., Wada, A., and Okamoto, M. (1998) Cloning of a membrane-spanning protein with epidermal growth factor-like repeat motifs from adrenal glomerulosa cells. Endocrinology 139, 3316• 3328
5. Jensen, C.H., Krogh, T.N., Højrup, P., Clausen, P.P., Skjødt, K., Larsson, L.I., Enghild, J.J., and Teisner, B. (1994) Protein structure of fetal antigen 1 (FA1). A novel circulating human epidermal-growth-factor-like protein expressed in neuroendocrine tumors and its relation to the gene products of dlk and pG2. Eur. J. Biochem. 225, 83• 92
6. Laborda, J., Sausville, E.A., Hoffman, T., and Notario, V. (1993) dlk, a putative mammalian homeotic gene differentially expressed in small cell lung carcinoma and neuroendocrine tumor cell line. J. Biol. Chem. 268, 3817• 3820
7. Lee, Y.L., Helman, L., Hoffman, T., and Laborda, J. (1995) dlk, pG2 and Pref-1 mRNAs encode similar proteins belonging to the EGF-like superfamily. Identification of polymorphic variants of this RNA. Biochim. Biophys. Acta 1261, 223• 232
8. Smas, C.M. and Sul, H.S. (1993) Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell 73, 725• 734
9. Floridon, C., Jensen, C.H., Thorsen, P., Nielsen, O., Sunde, L., Westergaard, J.G., Thomsen, S.G., and Teisner, B. (2000) Does fetal antigen 1 (FA1) identify cells with regenerative, endocrine and neuroendocrine potentials? A study of FA1 in embryonic, fetal, and placental tissue and in maternal circulation. Differentiation 66, 49• 59
10. Tanimizu, N., Nishikawa, M., Saito, H., Tsujimura, T., and Miyajima, A. (2003) Isolation of hepatoblasts based on the expression of Dlk/Pref-1. J. Cell Sci. 116, 1775• 1786
11. Schmidt, J.V., Matteson, P.G., Jones, B.K., Guan, X.J., and Tilghman, S.M. (2000) The Dlk1 and Gtl2 genes are linked and reciprocally imprinted. Genes Dev. 14, 1997• 2002
12. Laborda, J. (2000) The role of the epidermal growth factor-like protein dlk in cell differentiation. Histol. Histopathol. 15, 119• 129
13. Beatus, P. and Lendahl, U. (1999) Notch and neurogenesis. J. Neurosci. Res. 54, 125• 136
14. Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999) Notch signaling: cell fate control and signal integration in development. Science 284, 770• 776
15. Smas, C.M. and Sul, H.S. (1996) Characterization of Pref-1 and its inhibitory role in adipocyte differentiation. Int. J. Obes. Relat. Metab. Disord. 20 (Suppl. 3), S65• S72
16. Jordan, C.T. and Van Zant, G. (1998) Recent progress in identifying genes regulating hematopoietic stem cell function and fate. Curr. Opin. Cell Biol. 10, 716• 720
17. Moore, K.A., Pytowski, B., Witte, L., Hicklin, D., and Lemischka, I.R. (1997) Hematopoietic activity of a stromal cell transmembrane protein containing epidermal growth factor-like repeat motifs. Proc. Natl. Acad. Sci. USA 94, 4011• 4016
18. Tornehave, D., Jansen, P., Teisner, B., Rasmussen, H.B., Chemnitz, J., and Moscoso, G. (1993) Fetal antigen 1 (FA1) in the human pancreas: cell type expression, topological and quantitative variations during development. Anat. Embryol. (Berl.) 187, 335• 341
19. Tornehave, D., Jensen, C.H., Teisner, B., and Larsson, L.I. (1996) FA1 immunoreactivity in endocrine tumours and during development of the human fetal pancreas; negative correlation with glucagon expression. Histochem. Cell Biol. 106, 535• 542
20. Jensen, C.H., Teisner, B., Højrup, P., Rasmussen, H.B., Madsen, O.D., Nielsen, B., and Skjødt, K. (1993) Studies on the isolation, structural analysis and tissue localization of fetal antigen 1 and its relation to a human adrenal-specific cDNA, pG2. Hum. Reprod. 8, 635• 641
21. Cooper, M.J., Hutchins, G.M., Cohen, P.S., Helman, L.J., Mennie, R.J., and Israel, M.A. (1990) Human neuroblastoma tumor cell lines correspond to the arrested differentiation of chromaffin adrenal medullary neuroblasts. Cell Growth Differ. 1, 149• 159
22. Gaetano, C., Matsumoto, K., and Thiele, C.J. (1992) In vitro activation of distinct molecular and cellular phenotypes after induction of differentiation in a human neuroblastoma cell line. Cancer Res. 52, 4402• 4407
23. Hsiao, C.C., Huang, C.C., Sheen, J.M., Tai, M.H., Chen, C.M., Huang, L.L., and Chuang, J.H. (2005) Differential expression of delta-like gene and protein in neuroblastoma, ganglioneuroblastoma and ganglioneuroma. Mod. Pathol. 18, 656• 662
24. Yin, D., Xie, D., Sakajiri, S., Miller, C.W., Zhu, H., Popoviciu, M.L., Said, J.W., Black, K.L., and Koeffler, H.P. (2006) DLK1: increased expression in gliomas and associated with oncogenic activities. Oncogene 25, 1852• 1861
25. Harken Jensen, C., Drivsholm, L., Laursen, I., and Teisner, B. (1999) Elevated serum levels of fetal antigen 1, a member of the epidermal growth factor superfamily, in patients with small cell lung cancer. Tumour Biol. 20, 256• 262
26. Sakajiri, S., O'kelly, J., Yin, D., Miller, C.W., Hofmann, W.K., Oshimi, K., Shih, L.Y., Kim, K.H., Sul, H.S., Jensen, C.H., Teisner, B., Kawamata, N., and Koeffler, H.P. (2005) Dlk1 in normal and abnormal hematopoiesis. Leukemia 19, 1404• 1410
27. Altenberger, T., Bilban, M., Auer, M., Knosp, E., Wolfsberger, S., Gartner, W., Mineva, I., Zielinski, C., Wagner, L., and Luger, A. (2006) Identification of DLK1

variants in pituitary- and neuroendocrine tumors. Biochem. Biophys. Res. Commun. 340, 995• 1005
28. Fukuzawa, R., Heathcott, R.W., Morison, I.M., and Reeve, A.E. (2005) Imprinting, expression, and localisation of DLK1 in Wilms tumours. J. Clin. Pathol. 58, 145• 150
29. Fausto, N. and Campbell, J.S. (2003) The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech. Dev. 120, 117• 130
30. Shupe, T. and Petersen, B.E. (2005) Evidence regarding a stem cell origin of hepatocellular carcinoma. Stem Cell Rev. 1, 261• 264
31. Tanimizu, N., Tsujimura, T., Takahide, K., Kodama, T., Nakamura, K., and Miyajima, A. (2004) Expression of Dlk/Pref-1 defines a subpopulation in the oval cell compartment of rat liver. Gene Expr. Patterns 5, 209• 218
32. Singh, S.K., Clarke, I.D., Hide, T., and Dirks, P.B. (2004) Cancer stem cells in nervous system tumors. Oncogene 23, 7267• 7273
33. Al-Hajj, M., Becker, M.W., Wicha, M., Weissman, I., and Clarke, M.F. (2004) Therapeutic implications of cancer stem cells. Curr. Opin. Genet. Dev. 14, 43• 47
34. Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001) Stem cells, cancer, and cancer stem cells. Nature 414, 105• 111
35. Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., and Clarke, M.F. (2003) Prospective identification of tumorigenic breast cancer cells. Proc. Natl. Acad. Sci. USA 100, 3983• 3988
36. Al-Hajj, M. and Clarke, M.F. (2004) Self-renewal and solid tumor stem cells. Oncogene 23, 7274• 7282
37. Olempska, M., Eisenach, P.A., Ammerpohl, O., Ungefroren, H., Fandrich, F., and Kalthoff, H. (2007) Detection of tumor stem cell markers in pancreatic carcinoma cell lines. Hepatobiliary Pancreat. Dis. Int. 6, 92• 97
38. O'Brien, C.A., Pollett, A., Gallinger, S., and Dick, J.E. (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445, 106• 110
39. Singh, S.K., Hawkins, C., Clarke, I.D., Squire, J.A., Bayani, J., Hide, T., Henkelman, R.M., Cusimano, M.D., and Dirks, P.B. (2004) Identification of human brain tumour initiating cells. Nature 432, 396• 401
40. Collins, A.T., Berry, P.A., Hyde, C., Stower, M.J., and Maitland, N.J. (2005) Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res. 65, 10946• 10951
41. Ma, S., Chan, K.W., Hu, L., Lee, T.K., Wo, J.Y., Ng, I.O., Zheng, B.J., and Guan, X.Y. (2007) Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 132, 2542• 2556
42. Dezso, K., Halasz, J., Bisgaard, H.C., Paku, S., Turanyi, E., Schaff, Z., and Nagy, P. (2008) Delta-like protein (DLK) is a novel immunohistochemical marker for human hepatoblastomas. Virchows Arch. 452, 443• 448
43. Libbrecht, L. and Roskams, T. (2002) Hepatic progenitor cells in human liver diseases. Semin. Cell Dev. Biol. 13, 389• 396
44. Lowes, K.N., Brennan, B.A., Yeoh, G.C., and Olynyk, J.K. (1999) Oval cell numbers in human chronic liver diseases are directly related to disease severity. Am. J. Pathol. 154, 537• 541
45. Eleazar, J.A., Memeo, L., Jhang, J.S., Mansukhani, M.M., Chin, S., Park, S.M., Lefkowitch, J.H., and Bhagat, G. (2004) Progenitor cell expansion: an important source of hepatocyte regeneration in chronic hepatitis. J. Hepatol. 41, 983• 991
46. Jelnes, P., Santoni-Rugiu, E., Rasmussen, M., Friis, S.L., Nielsen, J.H., Tygstrup, N., and Bisgaard, H.C. (2007) Remarkable heterogeneity displayed by oval cells in rat and mouse models of stem cell-mediated liver regeneration. Hepatology 45, 1462• 1470
47. Lee, J.S., Heo, J., Libbrecht, L., Chu, I.S., Kaposi-Novak, P., Calvisi, D.F., Mikaelyan, A., Roberts, L.R., Demetris, A.J., Sun, Z., Nevens, F., Roskams, T., and Thorgeirsson, S.S. (2006) A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat. Med. 12, 410• 416
48. Huang, J., Zhang, X., Zhang, M., Zhu, J.D., Zhang, Y.L., Lin, Y., Wang, K.S., Qi, X.F., Zhang, Q., Liu, G.Z., Yu, J., Cui, Y., Yang, P.Y., Wang, Z.Q., and Han, Z.G. (2007) Up-regulation of DLK1 as an imprinted gene could contribute to human hepatocellular carcinoma. Carcinogenesis 28, 1094• 1103
49. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861• 872
50. Baladron, V., Ruiz-Hidalgo, M.J., Nueda, M.L., Diaz-Guerra, M.J., Garcia-Ramirez, J.J., Bonvini, E., Gubina, E., and Laborda, J. (2005) dlk acts as a negative regulator of Notch 1 activation through interactions with specific EGF-like repeats. Exp. Cell Res. 303, 343• 359
51. Qi, R., An, H., Yu, Y., Zhang, M., Liu, S., Xu, H., Guo, Z., Cheng, T., and Cao, X. (2003) Notch 1 signaling inhibits growth on human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. Cancer Res. 63, 8323• 8329

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JB Review
Liver stem/progenitor cells: their characteristics and regulatory mechanisms

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Liver stem cells give rise to both hepatocytes and bile duct epithelial cells also known as cholangiocytes. During liver development hepatoblasts emerge from the foregut endoderm and give rise to both cell types. Colony-forming cells are present in the liver primordium and clonally expanded cells differentiate into either hepatocytes or cholangiocytes depending on culture conditions, showing stem cell characteristics. The growth and differentiation of hepatoblasts are regulated by various extrinsic signals. For example, periportal mesenchymal cells provide a cue for bipotential hepatoblasts to become cholangiocytes, and mesothelial cells covering the parenchyma support the expansion of foetal hepatocytes by producing growth factors. The adult liver has an extraordinary capacity to regenerate, and after 70% hepatectomy the liver recovers its original mass by replication of the remaining hepatocytes without the activation of liver stem cells. However, in certain types of liver injury models, liver stem/progenitor-like cells, known as oval cells in rodents, proliferate around the portal vein, while the roles of such cells in liver regeneration remain a matter of debate. Clonogenic and bipotential cells are also present in the normal adult liver. In this minireview we describe recent studies on liver stem/progenitor cells by focusing on extracellular signals.

Keywords: cytokine/development/differentiation/hepatocyte/regeneration.

Abbreviations: 2-AAF, 2-acetylaminofluoren; AFP, alpha-fetoprotein; ALB, albumin; BMEL, bipotential mouse embryonic liver cell; CDE, choline-deficient, ethionine-supplemented; DDC, 3,5-diethoxycarbonyl-1,4-dihydro-collidine; Dlk, Delta-like protein 1; DPPIV, dipeptidyl peptidase IV; EpCAM, epithelial cell adhesion molecule; FGF, fibroblast growth factor; H-CFU-C, hepatic colony-forming unit in culture; MC, mesothelial cell; OSM, oncostatin M; PH, partial hepatectomy; STM, septum transversum mesenchyme; TNF, tumour necrosis factor; Wt1, Wilms' tumour 1.

The liver is a central organ for homeostasis owing to its numerous functions, including carbohydrate metabolism, glycogen storage, biosynthesis of various biochemical components including amino acids and nucleotides, lipid metabolism, urea synthesis, drug detoxification, production of plasma proteins and hormones, and destruction of erythrocytes. Because the liver is such an essential organ, liver diseases are often fatal. Liver insults such as hepatitis viruses, drugs, alcohol and genetic, metabolic and immune disorders can lead to steatosis, hepatitis, fibrosis, cirrhosis and cancer and liver disease is a major cause of death. The liver is also known as a unique organ that can regenerate, making it possible to transplant the liver from a living donor. However, the molecular mechanisms underlying organogenesis, maintenance, pathogenesis and regeneration of the liver are not well understood. As the liver is a large organ with a variety of functions, it has been used for many decades as a source to purify numerous enzymes for biochemical studies. By contrast, much progress has been made relatively recently in the characterization of each type of liver cell and analysis of their interactions. Those studies have been facilitated by new technologies such as genomics, mouse mutants and the development of various tools to isolate the cells of interest. In this review, we describe recent studies on liver stem/progenitor cells together with the environments that support their proliferation and differentiation during development and pathogenesis.

Liver architecture and liver stem cells

The liver is divided into lobules and each lobule consists of plates of hepatocytes lined by sinusoidal capillaries that radiate towards a central efferent vein (Fig. 1). Liver lobules are hexagonal and at each of six corners there is a portal triad of vessels consisting of a portal vein, hepatic artery and bile duct. Sinusoids are composed of liver-specific capillaries with fenestrated endothelial cells, hepatic stellate cells (Ito cells), liver-resident macrophages (Kupffer cells) and large granular lymphocytes (pit cells). The liver has a dual blood supply, namely, via the portal vein and the hepatic artery. The portal vein delivers the venous blood flowing from the intestines, pancreas and spleen. The hepatic artery supplies oxygen to the liver. The blood flows from a portal triad through a sinusoidal capillary to a central efferent vein. Hepatocytes are major parenchymal cells carrying out most of the metabolic functions and account for • 60% of the total liver cell population and 80% of the volume of the organ. Hepatocytes are highly polarized epithelial cells and

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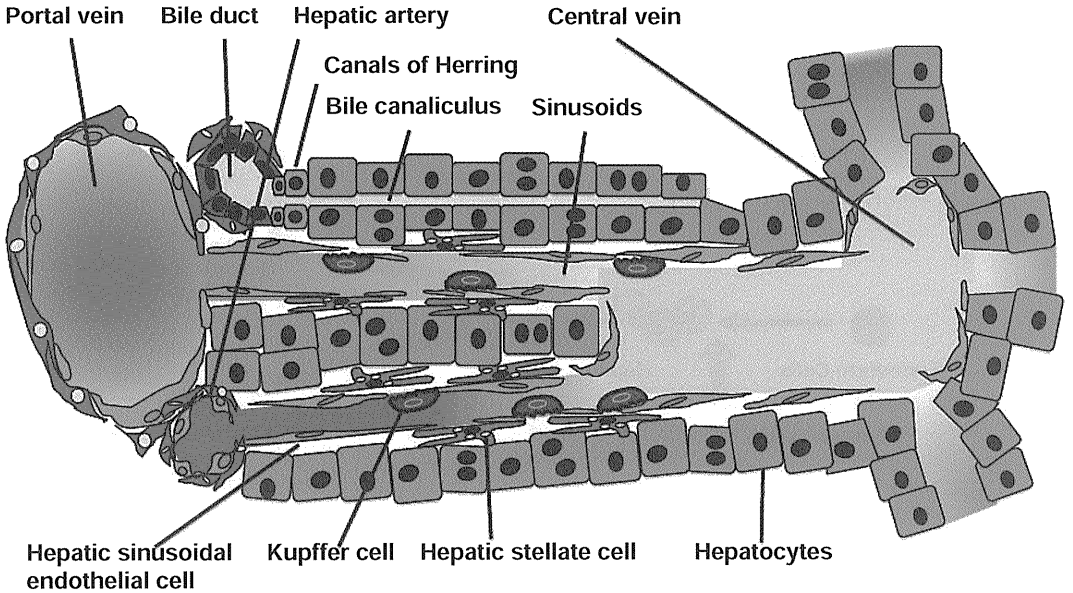

Fig. 1 Liver architecture. In the liver, blood flows from portal blood vessels through sinusoids to central efferent veins. Sinusoids are liver-specific capillaries with fenestrated endothelial cells, hepatic stellate cells (Ito cells) and blood cells such as liver-resident macrophages (Kupffer cells). Hepatocytes are highly polarized epithelial cells forming cords, and plates of hepatocytes are lined by sinusoidal capillaries that radiate towards a central efferent vein. Tight junctions formed between hepatocytes create a canaliculus that surrounds each hepatocyte. Bile salts produced in hepatocytes are excreted into canaliculi that are linked to bile ducts. The region that connects the bile canaliculus and the biliary tree is called 'canals of Hering'.

form cords. Their basolateral surfaces face fenestrated sinusoid endothelial cells, facilitating the transfer of materials between hepatocytes and blood flows. Tight junctions formed between hepatocytes create a canaliculus that surrounds each hepatocyte. Bile salts produced in hepatocytes are excreted into canaliculi that are linked to bile ducts at the portal triad. Bile ducts are formed by a specialized type of epithelial cell called a biliary epithelial cell or a cholangiocyte.

In general, stem cells are characterized by their ability to self-renew and differentiate to multiple lineages. As hepatocytes and cholangiocytes, the two types of liver epithelial cells, are derived from a common origin during organogenesis, those cells with the potential to proliferate and give rise to both types of liver epithelial cells are considered to be liver stem cells. Although there are many reports describing liver stem cells, the definitions of stem cells are rather vague in many of them. As it is not an easy task to distinguish stem cells from progenitors because of the difficulty of proving the unlimited self-renewal activity of stem cells in many situations, we use the term stem/progenitor cells to describe such cells in this review article.

The onset of liver development

Liver organogenesis begins at embryonic day (E) 8.5 in the mouse from the foregut endoderm. The ventral wall of the foregut endoderm faces the developing heart by approximately E8 and receives inductive signals for hepatic fate, such as fibroblast growth factor (FGF) from the heart (1• 3) and bone morphogenetic protein from the septum transversum mesenchyme (STM) (4). Wnt2b is expressed in the lateral plate mesoderm adjacent to the endoderm destined to be the liver and is essential for the onset of liver development in zebrafish (5). By these signals, hepatoblasts emerge from the foregut endoderm and migrate as cords into the surrounding STM (6, 7). Analysis of Flk1-deficient mouse embryos revealed that Flk1^{+} endothelial cells are required for proliferation of hepatoblasts (8). Because hepatoblasts proliferate and give rise to both hepatocytes and cholangiocytes as described below, they are considered to be embryonic liver stem/progenitor cells.

Identification and characterization of hepatoblasts

As cell sorting using antibodies is a powerful means to isolate and characterize a specific cell type, efforts have been made to search for specific cell surface antigens on hepatoblasts (Fig. 2). Kubota and Reid (9) showed that the RT1A1^{+} OX18^{low} ICAM-1^{+} fraction of E13 rat foetal liver contained hepatoblasts. Suzuki et al. (10) developed a single cell-based assay designated the hepatic colony-forming unit in culture (H-CFU-C) and showed that the CD45^{-} TER119^{-} c-Kit^{+} CD29^{+} CD49f^{+} and CD45^{-} TER119^{-} c-Kit^{+} c-Met^{+} CD49f$^{+/low}$ fraction of E13.5 mouse liver contained hepatic progenitor/stem cells. They also showed that CD45^{-} TER119^{-} c-Kit^{+} c-Met^{+} CD49f$^{+/low}$ cells of E11.5 mouse liver had high H-CFU-C potential and that clonally expanding cells reconstituted the liver, pancreas and intestine in vivo. On the other hand, Minguet et al. (11) reported that CD45^{-} TER119^{-} c-Kit^{low} cells in E11 mouse liver contained the earliest hepatic progenitors, also displaying features of

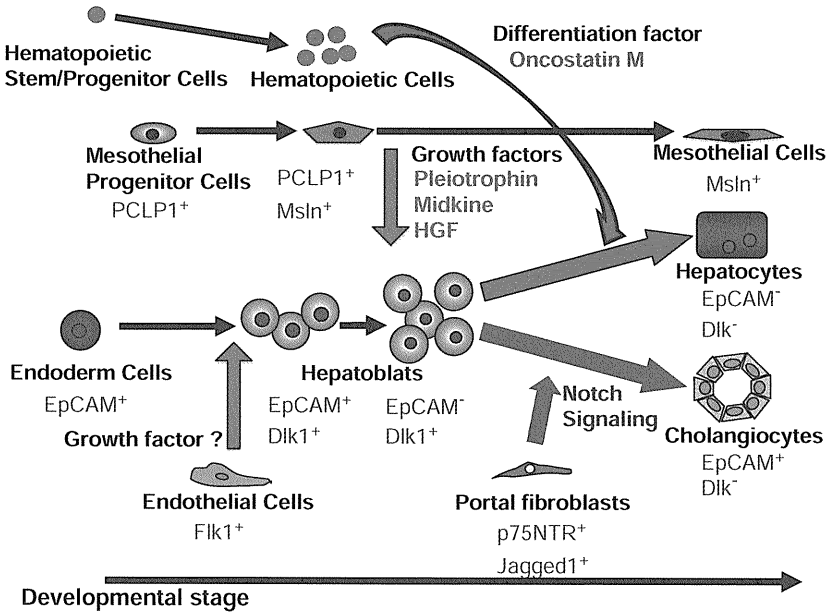

Fig. 2 Development of liver cells and expression of cell surface markers. The EpCAM$^+$ DLK1$^+$ hepatoblasts emerge from EpCAM$^+$ foregut endoderm cells and form liver primordium. Then, the hepatoblasts dramatically reduce the expression of EpCAM. EpCAM is upregulated again in biliary epithelial cell precursor cells around the portal vein, where p75NTR$^+$ Jagged1$^+$ portal fibroblasts interacted with hepatoblasts. PCLP1$^+$ mesothelial progenitor cells produce growth factors for hepatoblasts to proliferate. OSM secreted by haematopoietic cells induces hepatocytic differentiation of hepatoblasts.

liver-repopulating stem cells. Delta-like protein 1 (Dlk1), also known as Pref-1, was strongly expressed in liver buds as early as E10.5 in mice. Dlk1$^+$ cells isolated from E14.5 livers expressed albumin (ALB) and formed colonies composed of the hepatocyte and cholangiocyte lineages in the presence of hepatocyte growth factor and epidermal growth factor, indicating that liver stem cell activity is present in this population (12). As in mouse foetal liver, Dlk1 is also expressed strongly in human foetal liver (13, 14). Nierhoff et al. showed that murine foetal liver alpha-fetoprotein (AFP)$^+$/ALB$^+$ cells were positive for Dlk1 and E-cadherin and that purified E-cadherin$^+$ epithelial cells formed clusters in cell culture and differentiated along the hepatocytic lineage. Interestingly, AFP$^+$/E-cadherin$^+$ epithelial cells were Sca-1$^+$, but showed no expression of c-Kit. In order to examine their in vivo capacity, wild-type E12.5 mouse liver epithelial cells were transplanted into adult dipeptidyl peptidase IV (DPPIV) knockout mice, and DPPIV expression was used as a marker to discriminate the donor from recipient cells. This resulted in incorporation of the DPPIV$^+$ donor-derived cells into the hepatic parenchymal cords of the recipient liver, showing a repopulation and differentiation capacity of the E12.5 E-cadherin$^+$ cells (15).

Epithelial cell adhesion molecule (EpCAM) is expressed in HNF4a$^+$ hepatoblasts of liver buds as early as E9.5 in mice (Fig. 2). Colony-forming assays using sorted E11.5 liver cells revealed that the EpCAM$^+$ Dlk1$^+$ cell population contained in vitro colony-forming cells, indicating that liver stem cell activity is present in this population. EpCAM expression declined by E13.5 in mouse liver, while Dlk1 expression was sustained by E16.5 (16). In humans, Dan et al. (17) reported that multipotent progenitor cells derived from human foetal liver expressed EpCAM,and Schmelzer et al. (18) reported that pluripotent precursors of hepatoblasts expressed EpCAM and were located in ductal plates in human foetal liver. CD13 (aminopeptidase N) was detected on the cells of the Dlk1$^+$ hepatic stem/progenitor fraction. Colony formation assays revealed that hepatic stem/progenitor cells were enriched in the CD13$^+$ fraction, compared with the Dlk1$^+$ fraction, of non-haematopoietic cells in foetal liver (19).

Characteristics of foetal liver stem/progenitor cells

Dlk1$^+$ cells contain some clonogenic cells named hepatic progenitor proliferating on laminin that continuously proliferate on laminin-coated plates and differentiate to both hepatocytes and cholangiocytes depending on culture conditions, suggesting that they are liver stem cells (20). Bipotential cell lines, referred to as bipotential mouse embryonic liver cell (BMEL), were also obtained after a long latency in culture of foetal liver cells and they were shown to give rise to both hepatocytes and cholangiocytes in recipient mice, although the origin of BMEL was unknown (21). These cell lines are used to study the mechanisms of hepatocytic and/or cholangiocytic differentiation from liver stem cells.

In the past decade, a number of cell surface markers for foetal liver cells have been found and used to prospectively isolate and to localize them in the liver. While some studies used transplantation assays to

investigate the repopulation capacity, the ability to form a colony and differentiate to both lineages in vitro is a practical criterion to evaluate hepatoblasts in most of these studies. In the case of haematopoietic stem cells, a single purified stem cell can be shown to propagate and give rise to all kinds of haematopoietic cells for the long term in an irradiated recipient mouse, providing clear evidence for stemness in vivo, that is self-renewal ability and multi-lineage differentiation. By contrast, as liver repopulation assays require a large number of cells to be transplanted to demonstrate engraftment capacity, rigorous proof of stemness in vivo is difficult. Nonetheless, there is little doubt that hepatoblasts possess capacities of liver stem cells on the basis of numerous previous works as described above (9• 12, 15• 19).

Differentiation of hepatoblasts to cholangiocytes

Bile ducts are formed only around the portal vein, suggesting that regionally specific signals induce cholangiocytes from hepatoblasts. Indeed, two signalling pathways, TGFb/Activin and Notch, are specifically activated in hepatoblasts near the portal vein. TGFb2 and TGFb3 are predominantly expressed in the portal region (22), and the Onecut family of transcription factors, HNF6 (OC-1) and OC-2, promote expression of a2-macroglobulin and follistatin, inhibitors of the TGFb/Activin pathway, in the parenchymal region (23). Dlk1$^+$ hepatoblasts express Notch2, whereas p75NTR$^+$ periportal fibroblasts express Jagged-1 (24). Forced expression of Notch intracellular domain in Dlk1$^+$ hepatoblasts resulted in differentiation to cholangiocytes (25). These results strongly suggest that cholangiocyte differentiation is induced by Notch signalling in the periportal region. Although differentiation of hepatoblasts to cholangiocytes by TGFb and Notch signalling occurs in mid-gestation, surprisingly, hepatocytes turned to cholangiocytes and formed ectopic duct structures in the parenchyma by Notch activation after birth (26). These results indicate that not only hepatoblasts but also hepatocytes are competent to differentiate to cholangiocytes at least by the neonatal period (Fig. 2).

Immature cholangiocytes form a ductal plate, a single cell layer, around the portal vein. Tubular morphogenesis of bile ducts proceeds through the rearrangement of a single layer of the ductal plate. Recent studies on mice lacking Sox9, a transcription factor, or Notch 2 in the liver indicated the second wave of cholangiocyte differentiation adjacent to the initial single layer of the ductal plate, which was regulated by TGFb and Notch pathways and involved in tubular morphogenesis. In a model proposed on the basis of those studies, after the initial induction of cholangiocytes near the portal vein, cholangiocyte differentiation and tubular morphogenesis progress in parallel (22, 26• 30). However, the precise mechanisms of bile duct morphogenesis have not been completely understood.

In addition, studies using mutant mice have implicated transcription factors including HES1, HNF6, HNF1b, Tbx3, FoxA2 and A3, FoxM1b, Hex and Sall4 in bile duct differentiation and/or morphogenesis (31• 38) (Fig. 3). Although a network of these transcription factors and a link between transcription factors and Notch/TGFb pathways are being uncovered (39), studies on gene expression and histology of mutant mice are insufficient to understand how these factors regulate complicated processes of tubulogenesis. As an alternative approach, in vitro culture systems allowing hepatoblasts to form bile duct structures are helpful to understand the lineage commitment of hepatoblasts and tubular morphogenesis (22, 25, 40, 41).

Proliferation and differentiation of hepatocytes in foetal liver

At an early stage of hepatogenesis, endothelial cells contribute to the proliferation of hepatoblasts (8) and the vast majority of hepatoblasts become parenchymal hepatocytes at a later stage. The liver parenchyma is covered with the mesothelium consisting of the surface mesothelial cell (MC) layer, ALCAM$^+$ submesothelial cells and fibroblasts (42). At a later stage of hepatogenesis, MCs seem to contribute to the expansion of hepatoblasts (43). Foetal liver MCs are characterized by the expression of a sialomucin, PCLP1, and become adult liver MCs expressing mesothelin. Comparison of the gene expression profiles between foetal and adult MCs revealed that foetal PCLP1$^+$ MCs express various growth factors for hepatocytes such as Midkine and Pleiotrophin, and co-culture of Dlk1$^+$ foetal hepatocytes with PCLP1$^+$ foetal MCs in a transwell enhanced hepatocyte proliferation. Wilms' tumour 1 (Wt1) knockout mice were embryonic lethal, exhibiting impaired liver development. Cytokine production by Wt1 knockout MCs was reduced, while proliferation of Dlk1$^+$ cells from Wt1 knockout embryos was normal in a co-culture with wild-type MCs, indicating that defects in liver development of Wt1

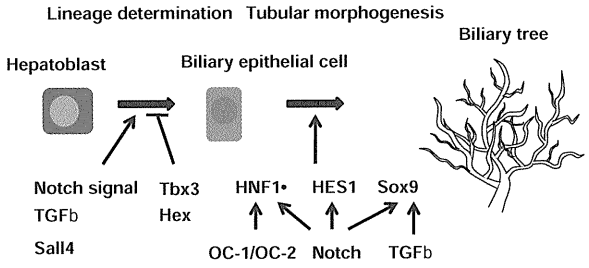

Fig. 3 Bile duct development. There are two steps in bile duct development. First, hepatoblasts are induced to differentiate to biliary epithelial cells around the portal vein. This step is promoted by TGFb and Notch signals as well as a transcription factor, Sall4, whereas it is inhibited by two transcription factors, Tbx3 and Hex. Biliary epithelial cells then undergo tubular morphogenesis and form the biliary tree. Three transcription factors, HNF1b, HES-1 and Sox9, are involved in tubular morphogenesis. The Notch signal is upstream of all the three transcription factors, whereas OC-1 and OC-2, and the TGFb signal are upstream of HNF1b and Sox9, respectively.

knockout mouse are due to MCs. MCs were also shown to delaminate and give rise to mesenchymal cells in the liver (44). These results indicate that the mesothelium is not only a protective sheet covering the liver parenchyma but also actively involved in liver organogenesis (Fig. 2).

Foetal liver is a major tissue for haematopoiesis, and hepatocytes acquire various metabolic functions at perinatal and postnatal stages. Mice lacking gp130, the common receptor subunit of the IL-6 family cytokines, develop liver with impaired functions, indicating that some of the IL-6 family cytokines are required for functional maturation of the liver (45). Oncostatin M (OSM), a member of the IL-6 family, strongly enhanced differentiation of foetal hepatocytes, while liver development is normal in OSM-deficient mice, suggesting that another member of the family may play a similar role. In the foetal liver, immigrating haematopoietic stem cells proliferate and produce numerous blood cells with the help of liver cells including hepatocytes and endothelial cells. Haematopoietic activity in foetal liver declines with hepatocyte differentiation (Fig. 2). As OSM is secreted from haematopoietic cells proliferating in the foetal liver and induces differentiation of hepatocytes, it is likely that OSM plays a role for coordination of liver development and haematopoiesis (46).

Adult liver stem/progenitor cells

Adult liver has a potential to regenerate under conditions of severe parenchymal loss, although hepatocytes and cholangiocytes are mitotically dormant under normal conditions. Hepatocytes themselves have a remarkable ability to self-replicate to restore liver mass (47) and are capable of at least 80 doublings by serial transplantation (48), allowing the liver to regenerate. Thus, the contribution of liver stem cells to regeneration after partial hepatectomy (PH) seems to be minimal if any. However, in liver injury that limits this pathway there is an accompanying expansion of a potential stem cell compartment in the periportal area, which is known as ductular reaction (49• 51) (Fig. 4). These proliferating epithelial cells are often referred to as oval cells in rodents because of their oval nucleus (52). Upon activation of oval cells, they expand into liver parenchyma from the portal area, and selective damage of the periportal zone reduces oval cell proliferation, supporting the notion that oval cells are derived from the periportal region, in particular canals of Hering that connect the bile canaliculus and the biliary tree (53). In addition, an extrahepatic origin of oval cells such as bone marrow was also suggested (54); however, the exact origin of oval cells still remains to be established. While oval cells have been most extensively studied in rodents, similar cells have been found in various human liver diseases, such as chronic viral hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease and fulminant hepatitis, and also implicated in tumourigenesis (55, 56). Oval cells express both ALB and cytokeratin 19, which are hepatocytic and cholangiocytic markers, respectively, and are believed to differentiate to hepatocytic and biliary lineages, similar to hepatoblasts in the embryonic liver.

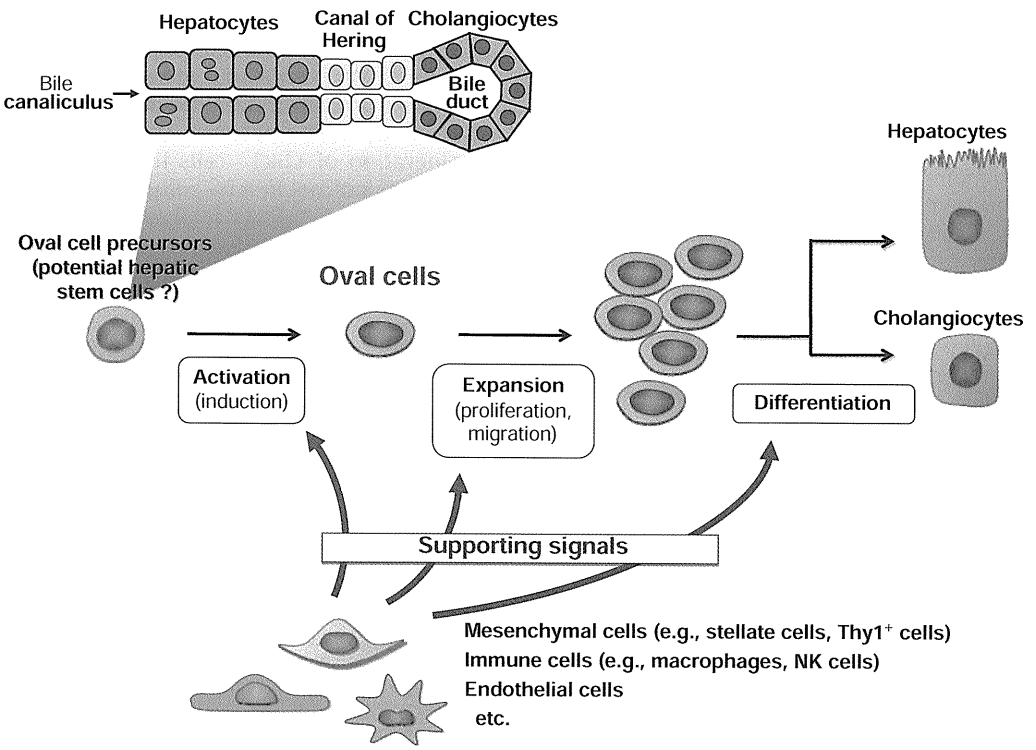

Fig. 4 Induction of oval cells. Oval cells and Thy1^{+} cells are simultaneously induced in severe liver damage conditions. The origin of oval cells is still under debate. FGF7 is produced by Thy1^{+} cells in response to liver damage conditions, and oval cells receive its signal.

Thus they are thought to be facultative stem/progenitor cells in the adult liver (Fig. 4).

The nature of oval cells as liver stem cells was debated in numerous reports of studies using various rodent models. The 2-acetylaminofluoren (2-AAF)/PH model, i.e. blocking hepatocyte proliferation by 2-AAF prior to PH, has been extensively used to characterize oval cells in rat (57, 58). However, the same procedure does not induce oval cells in mice, and alternative protocols such as a choline-deficient, ethionine-supplemented (CDE) diet and 3,5-diethoxycarbonyl-1,4-dihydro-collidine (DDC) diet have been developed to induce oval cells in mice (59• 61). Although the proliferating epithelial cells in the periportal region upon injury by various insults are collectively referred to as oval cells, it remains unclear whether or not the oval cells in different species by different protocols have common characteristics. A major problem in characterizing oval cells was the lack of appropriate cell surface markers to identify and isolate the oval cell compartment.

In the rat 2-AAF/PH model, Dabeva et al. reported that EpCAM$^+$ oval cells are bipotential adult hepatic progenitors (62, 63). Suzuki et al. (64) reported that CD133$^+$ cells isolated from DDC-treated mouse liver could form large colonies in culture. These large colony-forming cells gave rise to both hepatocytes and cholangiocytes, while maintaining undifferentiated cells by self-renewing cell divisions. In order to isolate and characterize mouse oval cells, Okabe et al. (65) searched for cell surface molecules expressed on oval cells in mouse fed DDC diet. EpCAM was expressed in both mouse normal cholangiocytes and oval cells, and its related protein TROP2 was expressed exclusively in oval cells, establishing TROP2 as a novel marker to distinguish oval cells from normal cholangiocytes (65). Some of the EpCAM$^+$ cells isolated from injured liver proliferate to form colonies in vitro, and the clonally expanded cells differentiate into hepatocytes and cholangiocytes, suggesting that the oval cell fraction contains potential liver stem cells.

Interestingly, such cells with liver stem cell characteristics are also found in EpCAM$^+$ cells of the normal liver. Intriguingly, comparison of the colony formation of EpCAM$^+$ cells between normal and injured livers revealed little difference in the frequency of potential liver stem cell activity between them, strongly suggesting that most of the proliferating mouse oval cells represent transit-amplifying cells rather than stem cells. Bipotential clonal cell lines can be obtained from the healthy liver of adult mice and participate in liver regeneration in severe combined immunedeficient mice expressing urokinase-type plasminogen by the ALB promoter, where they differentiate in clusters of hepatocytes and occasionally bile ducts (66). Kamiya et al. (67) found progenitor cells in the CD13$^+$ CD49f$^+$ CD133$^+$ subpopulation of non-haematopoietic cells derived from postnatal livers. These results demonstrate the existence, in normal adult mouse liver, of a pool of clonogenic cells that are (or can become) bipotential.

As mentioned above, oval cells are induced in liver with severe or chronic damage. Chronic injury conditions in the liver are usually associated with inflammation, and the roles of lymphocytes and inflammatory responses in oval cell regulation have also been suggested (68, 69). In accordance with this notion, several kinds of inflammatory cytokines, such as tumour necrosis factor (TNF)-a, lymphotoxin-b, interferon-g and IL-6, have been shown to modulate oval cell response (70, 71). Perhaps the best established inflammatory cytokine to be involved in oval cell response is a TNF family member ligand, TNF-like weak inducer of apoptosis (Tweak). Thus, transgenic mice overexpressing this cytokine in the liver exhibit periportal oval cell hyperplasia, while administration of a blocking anti-Tweak monoclonal antibody significantly reduced oval cell response in mice fed DDC diet (72). Furthermore, in mice lacking Fn14, the cognate receptor for Tweak, induction of oval cells was attenuated in both DDC diet and CDE diet models (72, 73). These inflammatory cytokines are considered to function as part of the innate immune system sensing damage to the tissue and serve as the earliest signals for triggering the process of liver regeneration (Fig. 4).

Mesenchymal cells such as stellate cells have long been suggested to physically interact with oval cells and thus considered to induce some signals in them (53). Recent studies using several rat and mouse models have demonstrated that a population of mesenchymal cells expressing thymus cell antigen-1 (Thy-1; also known as CD90) resides in close proximity to and expands in parallel with oval cells (74) (H. Takase, T. Itoh and A. Miyajima, unpublished observation). Furthermore, these Thy1$^+$ cells were found to express FGF7, and its cognate receptor FGFR2b was detected in oval cells. FGF7 knockout mice showed a defect in oval cell response, while overexpression of FGF7 in vivo in normal mouse liver led to induction and proliferation of cells with markers of oval cells in the periportal area. Together, these results strongly suggest that FGF7 plays a key role in adult liver stem/progenitor cell response as well as that the Thy1$^+$ cells may serve as the niche for oval cells by providing this cytokine (Takase, H., Itoh, T. and Miyajima A., unpublished observations). As a signal related to oval cell response, several recent studies have implicated the canonical Wnt/b-catenin pathway in oval cell regulation (75• 78) (Fig. 4). The Wnt/b-catenin pathway is well known to play important roles in stem cell regulation including self-renewal in various other organs and tissues, and also in carcinogenesis including liver tumours. In both rat and mouse models, expression of some Wnt ligands in damaged liver and concomitant activation of the b-catenin pathway in oval cells were observed. In conditional knockout mice lacking b-catenin in both hepatocytes and cholangiocytes, DDC diet-induced oval cell response in the liver was significantly reduced, although not completely abrogated. While several factors have been shown to be involved in oval cell response, the precise modes of their actions and their relationship are currently unclear and should be determined.

Concluding remarks

Traditionally, research on liver biology mostly relied on relatively crude cell separation methods based on cell density and centrifugation. In the last decade, identification of specific cell surface markers for each of the liver cell types, production of corresponding monoclonal antibodies and cell sorting techniques have together revolutionized the field and enabled us to perform much more detailed characterization of liver cells, particularly non-parenchymal cells including the stem/progenitor cells. It has also become possible to analyse the modes of interaction among different types of these cells in vivo by means of combinatorial use of specific markers/antibodies as well as in vitro with co-culture systems using the isolated viable cell populations. Elucidation of the molecular basis for the signals that regulate development, proliferation and differentiation of liver stem/progenitor cells should not only advance our understanding of the basic pathophysiology of the liver but also help to establish better protocols to generate mature hepatocytes and other liver cells in vitro for cell-based therapy, transplantation and drug discovery.

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Conflict of interest
None declared.

References

1. Douarin, N.M. (1975) An experimental analysis of liver development. Med. Biol. 53, 427• 455
2. Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J.R., and Zaret, K.S. (1996) Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes Dev. 10, 1670• 1682
3. Jung, J., Zheng, M., Goldfarb, M., and Zaret, K.S. (1999) Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science 284, 1998• 2003
4. Rossi, J.M., Dunn, N.R., Hogan, B.L., and Zaret, K.S. (2001) Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev. 15, 1998• 2009
5. Ober, E.A., Verkade, H., Field, H.A., and Stainier, D.Y. (2006) Mesodermal Wnt2b signalling positively regulates liver specification. Nature 442, 688• 691
6. Sosa-Pineda, B., Wigle, J.T., and Oliver, G. (2000) Hepatocyte migration during liver development requires Prox1. Nat. Genet. 25, 254• 255
7. Zaret, K. (1998) Early liver differentiation: genetic potentiation and multilevel growth control. Curr. Opin. Genet. Dev. 8, 526• 531
8. Matsumoto, K., Yoshitomi, H., Rossant, J., and Zaret, K.S. (2001) Liver organogenesis promoted by endothelial cells prior to vascular function. Science 294, 559• 563
9. Kubota, H. and Reid, L.M. (2000) Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. Proc. Natl. Acad. Sci. USA 97, 12132• 12137
10. Suzuki, A., Zheng, Y., Kondo, R., Kusakabe, M., Takada, Y., Fukao, K., Nakauchi, H., and Taniguchi, H. (2000) Flow-cytometric separation and enrichment of hepatic progenitor cells in the developing mouse liver. Hepatology 32, 1230• 1239
11. Minguet, S., Cortegano, I., Gonzalo, P., Martinez-Marin, J.A., de Andres, B., Salas, C., Melero, D., Gaspar, M.L., and Marcos, M.A. (2003) A population of c-Kit(low)(CD45/TER119)- hepatic cell progenitors of 11-day postcoitus mouse embryo liver reconstitutes cell-depleted liver organoids. J. Clin. Invest. 112, 1152• 1163
12. Tanimizu, N., Nishikawa, M., Saito, H., Tsujimura, T., and Miyajima, A. (2003) Isolation of hepatoblasts based on the expression of Dlk/Pref-1. J. Cell. Sci. 116, 1775• 1786
13. Floridon, C., Jensen, C.H., Thorsen, P., Nielsen, O., Sunde, L., Westergaard, J.G., Thomsen, S.G., and Teisner, B. (2000) Does fetal antigen 1 (FA1) identify cells with regenerative, endocrine and neuroendocrine potentials? A study of FA1 in embryonic, fetal, and placental tissue and in maternal circulation. Differentiation 66, 49• 59
14. Yanai, H., Nakamura, K., Hijioka, S., Kamei, A., Ikari, T., Ishikawa, Y., Shinozaki, E., Mizunuma, N., Hatake, K., and Miyajima, A. (2010) Dlk-1, a cell surface antigen on foetal hepatic stem/progenitor cells, is expressed in hepatocellular, colon, pancreas and breast carcinomas at a high frequency. J. Biochem. 148, 85• 92
15. Nierhoff, D., Ogawa, A., Oertel, M., Chen, Y.Q., and Shafritz, D.A. (2005) Purification and characterization of mouse fetal liver epithelial cells with high in vivo repopulation capacity. Hepatology 42, 130• 139
16. Tanaka, M., Okabe, M., Suzuki, K., Kamiya, Y., Tsukahara, Y., Saito, S., and Miyajima, A. (2009) Mouse hepatoblasts at distinct developmental stages are characterized by expression of EpCAM and DLK1: drastic change of EpCAM expression during liver development. Mech. Dev. 126, 665• 676
17. Dan, Y.Y., Riehle, K.J., Lazaro, C., Teoh, N., Haque, J., Campbell, J.S., and Fausto, N. (2006) Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages. Proc. Natl. Acad. Sci. USA 103, 9912• 9917
18. Schmelzer, E., Zhang, L., Bruce, A., Wauthier, E., Ludlow, J., Yao, H.L., Moss, N., Melhem, A., McClelland, R., Turner, W., Kulik, M., Sherwood, S., Tallheden, T., Cheng, N., Furth, M.E., and Reid, L.M. (2007) Human hepatic stem cells from fetal and postnatal donors. J. Exp. Med. 204, 1973• 1987
19. Kakinuma, S., Ohta, H., Kamiya, A., Yamazaki, Y., Oikawa, T., Okada, K., and Nakauchi, H. (2009) Analyses of cell surface molecules on hepatic stem/progenitor cells in mouse fetal liver. J. Hepatol. 51, 127• 138
20. Tanimizu, N., Saito, H., Mostov, K., and Miyajima, A. (2004) Long-term culture of hepatic progenitors derived from mouse Dlk• hepatoblasts. J. Cell. Sci. 117, 6425• 6434
21. Strick-Marchand, H., Morosan, S., Charneau, P., Kremsdorf, D., and Weiss, M.C. (2004) Bipotential mouse embryonic liver stem cell lines contribute to liver regeneration and differentiate as bile ducts and hepatocytes. Proc. Natl. Acad. Sci. USA 101, 8360• 8365
22. Antoniou, A., Raynaud, P., Cordi, S., Zong, Y., Tronche, F., Stanger, B.Z., Jacquemin, P., Pierreux, C.E., Clotman, F., and Lemaigre, F.P. (2009) Intrahepatic bile

ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 136, 2325• 2333
23. Clotman, F., Jacquemin, P., Plumb-Rudewiez, N., Pierreux, C.E., Van der Smissen, P., Dietz, H.C., Courtoy, P.J., Rousseau, G.G., and Lemaigre, F.P. (2005) Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. Genes Dev. 19, 1849• 1854
24. Suzuki, K., Tanaka, M., Watanabe, N., Saito, S., Nonaka, H., and Miyajima, A. (2008) p75 Neurotrophin receptor is a marker for precursors of stellate cells and portal fibroblasts in mouse fetal liver. Gastroenterology 135, 270• 281 e273
25. Tanimizu, N. and Miyajima, A. (2004) Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. J. Cell. Sci. 117, 3165• 3174
26. Zong, Y., Panikkar, A., Xu, J., Antoniou, A., Raynaud, P., Lemaigre, F., and Stanger, B.Z. (2009) Notch signaling controls liver development by regulating biliary differentiation. Development 136, 1727• 1739
27. Geisler, F., Nagl, F., Mazur, P.K., Lee, M., Zimber-Strobl, U., Strobl, L.J., Radtke, F., Schmid, R.M., and Siveke, J.T. (2008) Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology 48, 607• 616
28. Lozier, J., McCright, B., and Gridley, T. (2008) Notch signaling regulates bile duct morphogenesis in mice. PLoS One 3, e1851
29. Sparks, E.E., Huppert, K.A., Brown, M.A., Washington, M.K., and Huppert, S.S. (2010) Notch signaling regulates formation of the three-dimensional architecture of intrahepatic bile ducts in mice. Hepatology 51, 1391• 1400
30. Tchorz, J.S., Kinter, J., Muller, M., Tornillo, L., Heim, M.H., and Bettler, B. (2009) Notch2 signaling promotes biliary epithelial cell fate specification and tubulogenesis during bile duct development in mice. Hepatology 50, 871• 879
31. Clotman, F., Lannoy, V.J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G.G., and Lemaigre, F.P. (2002) The onecut transcription factor HNF6 is required for normal development of the biliary tract. Development 129, 1819• 1828
32. Coffinier, C., Gresh, L., Fiette, L., Tronche, F., Schutz, G., Babinet, C., Pontoglio, M., Yaniv, M., and Barra, J. (2002) Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. Development 129, 1829• 1838
33. Hunter, M.P., Wilson, C.M., Jiang, X., Cong, R., Vasavada, H., Kaestner, K.H., and Bogue, C.W. (2007) The homeobox gene Hhex is essential for proper hepatoblast differentiation and bile duct morphogenesis. Dev. Biol. 308, 355• 367
34. Kodama, Y., Hijikata, M., Kageyama, R., Shimotohno, K., and Chiba, T. (2004) The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology 127, 1775• 1786
35. Krupczak-Hollis, K., Wang, X., Kalinichenko, V.V., Gusarova, G.A., Wang, I.C., Dennewitz, M.B., Yoder, H.M., Kiyokawa, H., Kaestner, K.H., and Costa, R.H. (2004) The mouse Forkhead Box m1 transcription factor is essential for hepatoblast mitosis and development of intrahepatic bile ducts and vessels during liver morphogenesis. Dev. Biol. 276, 74• 88
36. Li, Z., White, P., Tuteja, G., Rubins, N., Sackett, S., and Kaestner, K.H. (2009) Foxa1 and Foxa2 regulate bile duct development in mice. J. Clin. Invest. 119, 1537• 1545
37. Oikawa, T., Kamiya, A., Kakinuma, S., Zeniya, M., Nishinakamura, R., Tajiri, H., and Nakauchi, H. (2009) Sall4 regulates cell fate decision in fetal hepatic stem/progenitor cells. Gastroenterology 136, 1000• 1011
38. Suzuki, A., Sekiya, S., Buscher, D., Izpisua Belmonte, J.C., and Taniguchi, H. (2008) Tbx3 controls the fate of hepatic progenitor cells in liver development by suppressing p19ARF expression. Development 135, 1589• 1595
39. Raynaud, P., Carpentier, R., Antoniou, A., and Lemaigre, F.P. (2011) Biliary differentiation and bile duct morphogenesis in development and disease. Int. J. Biochem. Cell Biol. 43, 245• 256
40. Tanimizu, N., Miyajima, A., and Mostov, K.E. (2007) Liver progenitor cells develop cholangiocyte-type epithelial polarity in three-dimensional culture. Mol. Biol. Cell 18, 1472• 1479
41. Tanimizu, N., Miyajima, A., and Mostov, K.E. (2009) Liver progenitor cells fold up a cell monolayer into a double-layered structure during tubular morphogenesis. Mol. Biol. Cell 20, 2486• 2494
42. Asahina, K., Tsai, S.Y., Li, P., Ishii, M., Maxson, R.E. Jr, Sucov, H.M., and Tsukamoto, H. (2009) Mesenchymal origin of hepatic stellate cells, submesothelial cells, and perivascular mesenchymal cells during mouse liver development. Hepatology 49, 998• 1011
43. Onitsuka, I., Tanaka, M., and Miyajima, A. (2010) Characterization and functional analyses of hepatic mesothelial cells in mouse liver development. Gastroenterology 138, 1525• 1535, 1535 e1521• 1526
44. Ijpenberg, A., Perez-Pomares, J.M., Guadix, J.A., Carmona, R., Portillo-Sanchez, V., Macias, D., Hohenstein, P., Miles, C.M., Hastie, N.D., and Munoz-Chapuli, R. (2007) Wt1 and retinoic acid signaling are essential for stellate cell development and liver morphogenesis. Dev. Biol. 312, 157• 170
45. Kamiya, A., Kinoshita, T., Ito, Y., Matsui, T., Morikawa, Y., Senba, E., Nakashima, K., Taga, T., Yoshida, K., Kishimoto, T., and Miyajima, A. (1999) Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. EMBO J. 18, 2127• 2136
46. Kinoshita, T., Sekiguchi, T., Xu, M.J., Ito, Y., Kamiya, A., Tsuji, K., Nakahata, T., and Miyajima, A. (1999) Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. Proc. Natl. Acad. Sci. USA 96, 7265• 7270
47. Michalopoulos, G.K. (2010) Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. Am. J. Pathol. 176, 2• 13
48. Overturf, K., al-Dhalimy, M., Ou, C.N., Finegold, M., and Grompe, M. (1997) Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes. Am. J. Pathol. 151, 1273• 1280
49. Alison, M.R., Golding, M.H., and Sarraf, C.E. (1996) Pluripotential liver stem cells: facultative stem cells located in the biliary tree. Cell Prolif. 29, 373• 402
50. Roskams, T.A., Theise, N.D., Balabaud, C., Bhagat, G., Bhathal, P.S., Bioulac-Sage, P., Brunt, E.M., Crawford, J.M., Crosby, H.A., Desmet, V., Finegold, M.J., Geller, S.A., Gouw, A.S., Hytiroglou, P., Knisely, A.S., Kojiro, M., Lefkowitch, J.H., Nakanuma, Y., Olynyk, J.K., Park, Y.N., Portmann, B., Saxena, R., Scheuer, P.J., Strain, A.J., Thung, S.N., Wanless, I.R., and West, A.B. (2004) Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology 39, 1739• 1745
51. Theise, N.D., Saxena, R., Portmann, B.C., Thung, S.N., Yee, H., Chiriboga, L., Kumar, A., and Crawford, J.M.