Supplementary Materials and Methods

Antibodies

Goat anti-mouse PCLP1 polyclonal Ab and rabbit anti-mouse Ki67 polyclonal Ab used for IHC were purchased from R&D Systems, Inc. Phycoerythrin-conjugated or biotinylated anti-mouse PCLP1 monoclonal Ab (clone 10B9) used for flow cytometry was supplied from Medical & Biological Laboratories, Co, Ltd (Nagoya, Japan). Biotinylated anti-mouse Msln monoclonal Ab (clone B35) for both IHC and flow cytometry was prepared as described previously.[1] Anti-mouse delta-like 1 homologue (Dlk1) monoclonal Ab[2] was biotinylated by ECL Protein Biotinylation Module (GE Healthcare, Tokyo, Japan) according to the manufacturer's protocol. Other fluorescently labeled Abs and anti-FcγR Ab used for flow cytometry were purchased from Pharmingen (San Diego, CA). To detect signals of biotinylated Abs in flow cytometry, allophycocyamin-conjugated streptavidin (BioLegend, San Diego, CA) was used.

IHC

Whole embryos or livers dissected from animals were frozen and cryosectioned into 6-μm slices. Sections were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 minutes at room temperature, followed by incubation with each primary Ab. Sections were incubated with fluorescently labeled secondary antibodies to detect signals by fluorescence microscopy. Ki67 immunocytochemistry was performed using PCLP1^{high} MCs sorted from E13.5 livers by a cell sorter. Cytospin samples were fixed and immunostained using Ki67 Ab as described previously.

Quantitative RT-PCR Analysis

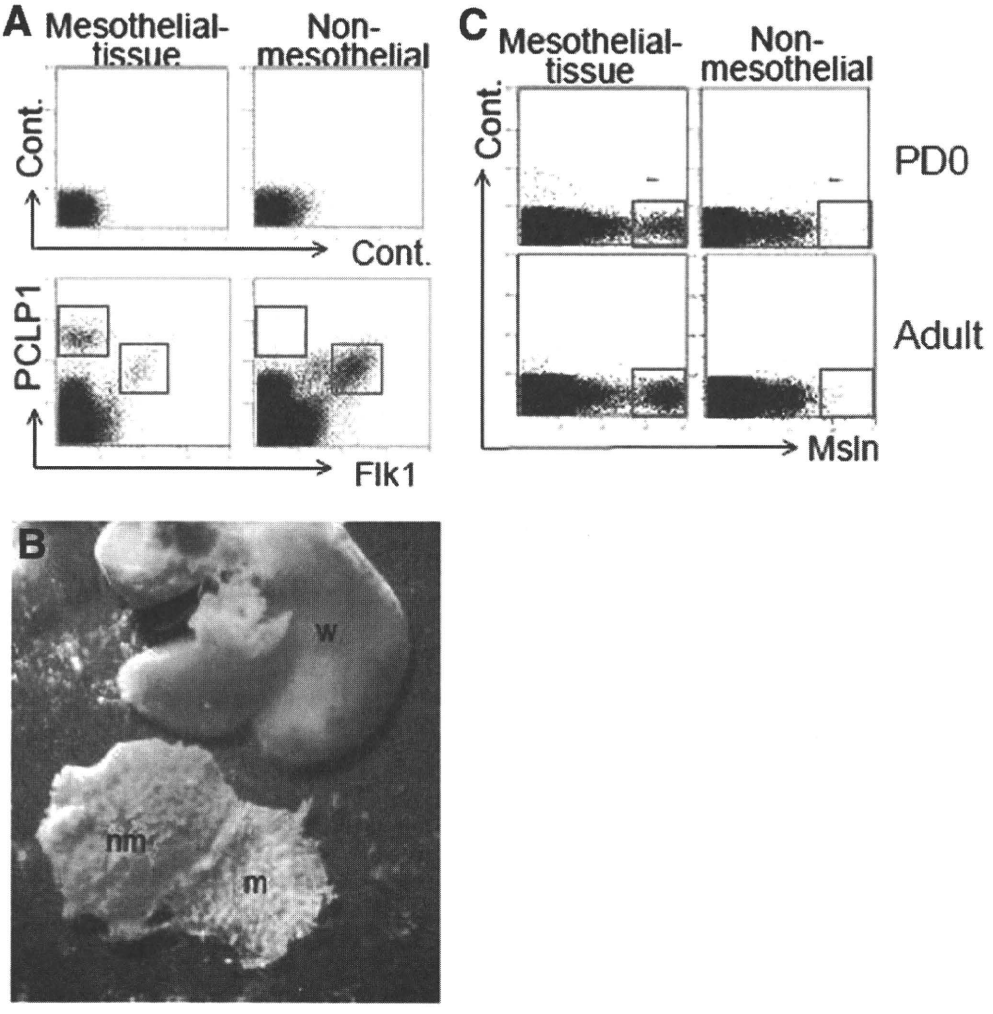
Total RNA was extracted from cells by using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand complementary DNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and was used as a template for PCR amplification. The primer sequences used are as follows: *GAPDH*, 5′-TGAACGGGAAGCTCACTGG-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′; *Msln*, 5′-CTATCCTGAGTCCCTGATCCA-3′ and 5′-TCGCCTGAGCATTCATCTTT-3′; *HGF*, 5′-CCCGAGAACTTCAAATGCAA-3′ and 5′-TATGACGGTGTAAATCCTCCA-3′; *Ptn*, 5′-GGAAGAAGCAGTTTGGAGCTG-3′ and 5′-GGCGGTATTGAGGTCACATTC-3′; *Mdk*, 5′-TGATGGGAGCACTGGCAC-3′ and 5′-CATTGTACCGCGCCTTCTT-3′; *PCLP1*, 5′-GCAAGAGCGGTGACAGTTTTA-3′ and 5′-AGTTGTCAGTGCTGGGCGT-3′; *Msln*, 5′-CTATCCTGAGTCCCTGATCCA-3′ and 5′-TCGCCTGAGCATTCATCTTT-3′; *WT1*, 5′-CAGATGAACCTAGGAGCTACCTTAAA-3′ and 5′-CGTGGTTGCTCTGCCCTTCT-3′; *Raldh2*, 5′-CATGGTATCCTCCGCAATG-3′ and 5′-GCGCATTTAAGGCATTGTAAC-3′. The real-time PCR reactions were performed using LightCycler (Roche Diagnostics KK, Toyko, Japan) according to the manufacturer's protocol.

In Vitro Colony Formation Assay

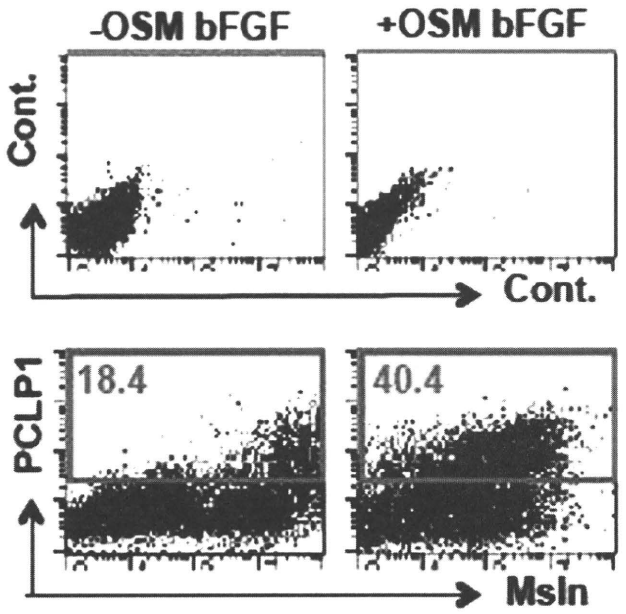
MCs were prepared by sorting of Flk1^{-}PCLP1^{high} cells using EPICS ALTRA from E12.5 livers or by sorting of Msln^{+} cells using autoMACS or EPICS ALTRA from E18.5, PD7, or adult livers. MCs (1×10^3 cells) were then inoculated into each well of a type IV collagen-coated 6-well plate (AGC Techno Glass, Chiba, Japan) and cultured in α-minimum essential medium (Invitrogen) containing 10% fetal bovine serum (JRH Biosciences, Tokyo, Japan) and 50 nmol/L mercaptoethanol (Invitrogen). After 6 days of culture, the plates were stained with Giemsa solution (Merck, Damstadt, Germany) to visualize the cells and colonies were counted under the microscope. Averages of 3 wells from each sample were used to evaluate cell proliferation.

References

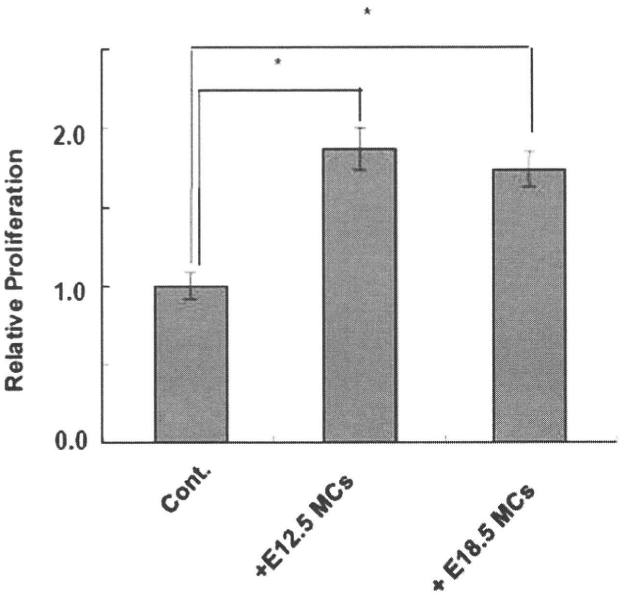
1. Rump A, Morikawa Y, Tanaka M, et al. Binding of ovarian cancer antigen CA125/MUC16 to mesothelin mediates cell adhesion. J Biol Chem 2004;279:9190–9198.
2. Tanaka M, Okabe M, Suzuki K, et al. Mouse hepatoblasts at distinct developmental stages are characterized by expression of EpCAM and DLK1: drastic change of EpCAM expression during liver development. Mech Dev 2009;126:665–676.



Supplementary Figure 1. (*A*) FCM of E16.5 liver cells with anti-PCLP1 and anti-Flk1 Abs. *Red* and *green lines* in *lower panels* indicate PCLP1^{high} and PCLP1^{med} cell populations, respectively. Note that Flk1^{-}PCLP1^{high} cells were present exclusively in the mesothelial tissue, while Flk1^{+}PCLP1^{med} cells were detected mainly in the non-mesothelial tissue. (*B*) Appearance of surgically separated adult liver mesothelial tissue (m) and non-mesothelial tissue (nm). w, non-separated whole liver. (*C*) FCM with anti-Msln Ab using neonatal (PD0) or adult liver cells. *Red lines* in each *panel* indicate Msln^{+} cell population. Note that the cells highly expressing Msln are exclusively detected in the surgically separated mesothelial tissue in PD0 and adult livers.



Supplementary Figure 2. FCM of cultured MCs with anti-PCLP1 and anti-MsIn Abs. After first passage of cultured E12.5 Flk1^{-}PCLP1^{high} cells in the presence of OSM and bFGF, the cells were incubated for additional 6 days in the presence or absence of the cytokines, followed by FCM. *Red lines* and numbers in *lower panels* indicate PCLP1^{+} cell populations and their percentages, respectively.

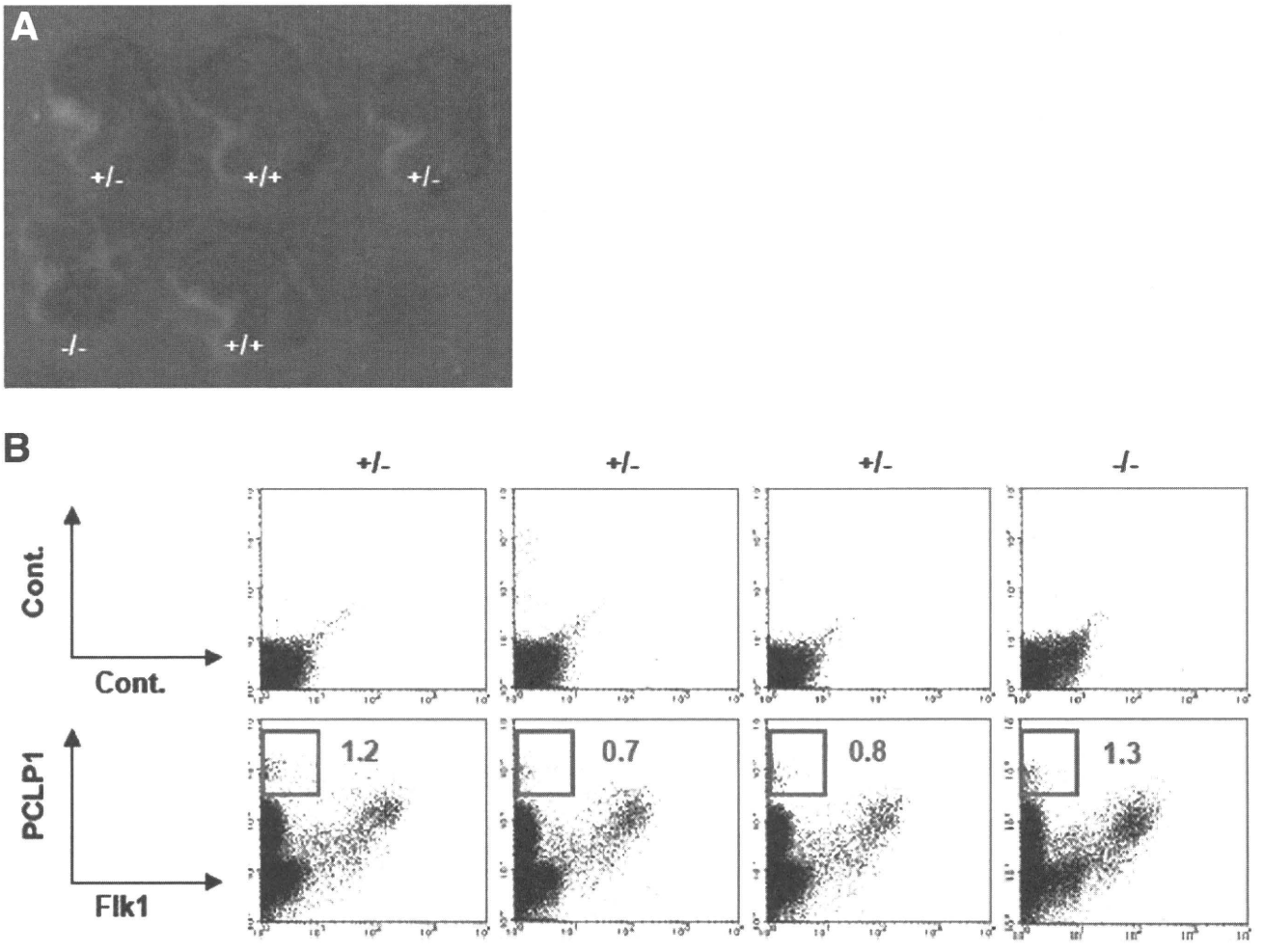


Supplementary Figure 3. Proliferation of E14.5 Dlk1^{+} cells cultured with in vitro expanded MCs from E12.5 or E18.5 livers. Growth of hepatocytes was measured by WST-1 assay after 3 days of coculture. Averages of 3 wells for each sample are shown. *$P < .005$; Student *t* test.

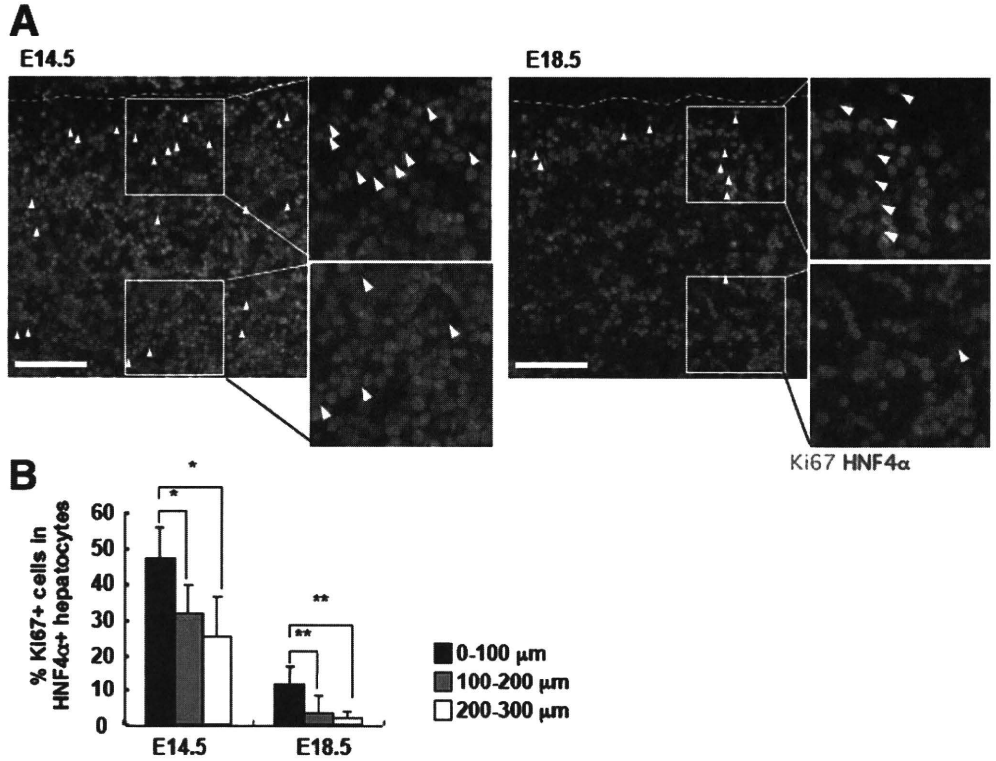
Supplementary Table 1. Growth Factors Preferentially Expressed in Immature MCs

Genbank	Gene symbol	Description	E12.5 PCLP1highMsIn^{-}	Adult MsIn^{+}	E12.5/Adult Ratio
AV240088	Fgf5	Fibroblast growth factor 5	413.0	1.0	>100.0
NM_010514	Igf2	Insulin-like growth factor 2	226.3	1.4	>100.0
BC002064	Ptn	Pleiotrophin	2129.3	18.1	80.0
U50279	Vegfa	Vascular endothelial growth factor A	79.7	0.8	68.3
AV269710	Angpt4	Angiopoietin 4	124.7	2.0	42.6
M34328	Mdk	Midkine	9680.6	158.6	41.5
AF020737	Fgf13	Fibroblast growth factor 13	198.8	4.1	32.9
NM_019971	Pdgfc	Platelet-derived growth factor, C polypeptide	2805.1	58.1	32.8
NM_008243	Mst1	Macrophage stimulating 1 (hepatocyte growth factor-like)	75.0	1.6	31.5
NM_010514	Igf2	Insulin-like growth factor 2	12,753.5	285.0	30.4
NM_019971	Pdgfc	Platelet-derived growth factor, C polypeptide	3830.3	109.1	23.9
BC002064	Ptn	Pleiotrophin	878.0	26.0	22.9
NM_009704	Areg	Amphiregulin	20.2	0.7	19.7
NM_009521	Wnt3	Wingless-related MMTV integration site 3	5.5	0.2	16.0
AU015375	Bmp15	Bone morphogenetic protein 15	12.7	0.6	15.5
NM_010201	Fgf14	Fibroblast growth factor 14	14.9	1.0	10.1
AV032115	Bmp5	Bone morphogenetic protein 5	133.8	9.6	9.4
AB073819	Wnt9b	Wingless-type MMTV integration site 9B	14.8	1.2	8.6
BB476818	Hgf	Hepatocyte growth factor	143.2	12.9	7.5
AB016516	Fgf5	Fibroblast growth factor 5	26.6	2.5	7.3
AK003506	Il17b	Interleukin 17B	35.1	3.6	6.6
NM_009505	Vegfa	Vascular endothelial growth factor A	660.0	75.6	5.9
NM_021782	Il21	Interleukin 21	15.7	1.9	5.6
NM_021380	Il20	Interleukin 20	67.4	8.8	5.2
BM210179	Bmp8b	Bone morphogenetic protein 8b	22.9	3.0	5.2

NOTE. Microarray analysis was performed using E12.5 PCLP1^{high}MsIn^{-} immature MCs and adult mature MsIn^{+} MCs. The list shows growth factors whose expression in E12.5 MCs is more than 5 times higher than adult MCs.



Supplementary Figure 4. (*A*) The morphology of E13.5 livers from littermates with each genotype. Note that the liver of $WT1^{-/-}$ embryo is smaller than those of the other littermates. (*B*) FCM of E13.5 liver cells from each littermate with anti-Flk1 and anti-PCLP1 Abs. The genotypes are indicated above each *panel*. *Red lines* and numbers in the *lower panels* indicate $Flk\text{-}1^{-}PCLP1^{high}$ cells and its percentages, respectively. Note that $Flk1^{-}PCLP1^{high}$ cells are detected in $WT1^{-/-}$ FLs as well as $WT1^{+/-}$ littermates.

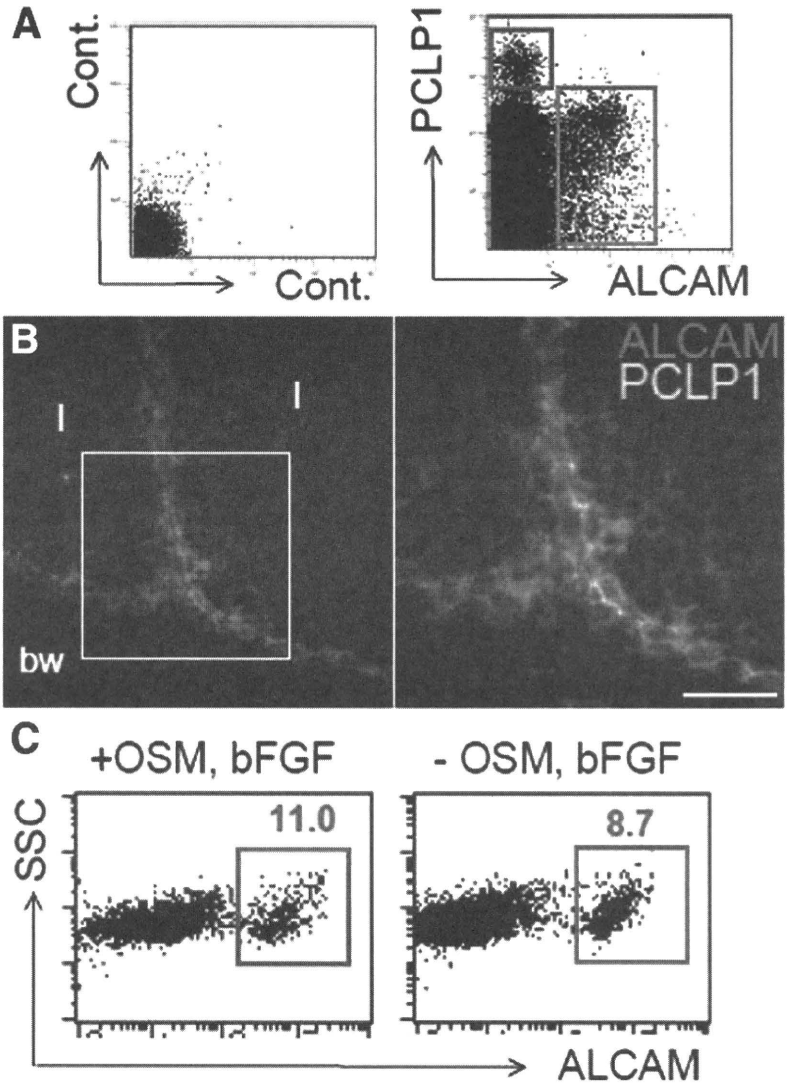


Supplementary Figure 5. Distribution of proliferating hepatocytes in the developing liver (*A*) immunostaining of E12.5 and E18.5 FL sections with anti-Ki67 (*green*) and anti-HNF4α (*red*) Abs. The *dotted lines* in the *left panels* indicate MC layer. Higher magnified images of *boxed areas* are shown in the *right*. The *arrowheads* indicate Ki67+HNF4α+ cells. Note that Ki67$^+$HNF4α$^+$ cells are observed more abundantly in the peripheral regions than in the central regions of hepatic lobes. *Scale bars*, 80 μm. (*B*) The percentages of Ki67$^+$ cells in HNF4α$^+$ cells in each 100 × 300 μm^2 area at 0–100, 100–200, and 200–300 μm distance from the MC layer. Microscopic views of 300 μm × 300 μm were used for analyses (E14.5, n = 5; E18.5, n = 12). *$P < .05$; **$P < .005$; Student *t* test.

Supplementary Table 2. The Number of HNF4α$^+$ cells Used for Analysis

	Number of HNF4α$^+$ cell in each 100 × 300 μm^2 area		
	0–100 μm	100–200 μm	200–300 μm
E14.5 (n* = 5)	38.4 ± 8.3 (47.2 ± 8.7)	38.8 ± 9.5 (32.1 ± 8.0)	35.2 ± 5.0 (25.4 ± 11.3)
E18.5 (n* = 12)	56.9 ± 10.9 (11.5 ± 5.4)	58.4 ± 7.0 (3.2 ± 5.0)	52.9 ± 8.0 (1.7 ± 2.0)

NOTE. The number of HNF4α$^+$ cells in each 100 × 300 μm^2 area at 0–100, 100–200, and 200–300 μm distance from the MC layer was counted for Supplementary Figure 4*B*. The percentages of Ki67$^+$ cells in HNF4α$^+$ cells are shown in parentheses. n*, number of 300 μm × 300 μm views.



Supplementary Figure 6. (*A*) FCM of E12.5 liver cells using fluorescently labeled Abs against ALCAM and PCLP1. *Red* and *green lines* in the *right panel* indicate PCLP1^{high} cells and ALCAM^{+} cells, respectively. Note that PCLP1^{high} cells are negative for ALCAM. (*B*) IHC on E13.5 liver sections with anti-ALCAM (*red*) and anti-PCLP1 (*green*) Abs. Higher magnification image of the *boxed region* in the *left panel* is shown in the *right panel*. Note that PCLP1^{+} MC layers and ALCAM^{+} submesothelial layers are clearly distinguishable. l, lobe; bw, body wall. *Scale bar*, 40 μm. (*C*) FCM of cultured MCs using fluorescently labeled Abs against ALCAM. PCLP1^{high} cells sorted from E12.5 FL were cultured in the presence of OSM and bFGF, and further cultured for 3 days in the presence or absence of OSM and bFGF. Note that ALCAM^{-}PCLP1^{high} immature MCs give rise to ALCAM^{+} cells in vitro.

IκBη, a nuclear IκB protein, positively regulates the NF-κB–mediated expression of proinflammatory cytokines

Shumpei Yamauchi, Hiroaki Ito[1], and Atsushi Miyajima[2]

Laboratory of Cell Growth and Differentiation, Institute of Molecular and Cellular Bioscience, University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan

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NF-κB is a key mediator for inducible transcription of various proinflammatory genes in innate immune responses, and its activity is strictly regulated by several IκB proteins. Although signaling pathways leading from pattern recognition receptors to NF-κB's activation in the cytoplasm have been studied extensively, the detail regulatory mechanisms of NF-κB–mediated transcriptional activity in the nucleus still remain unclear. Here we describe a unique member of the nuclear IκB protein family, IκBη. In a gene expression analysis of dendritic cells, we found a unique gene encoding an uncharacterized protein with ankyrin repeats. As it was structurally related to the IκB family, the protein was named "IκBη" and further characterized in the innate immune response. IκBη was widely expressed in various tissues and predominantly located in the nucleus. Moreover, biochemical analysis showed that IκBη associated with the p50 subunit of NF-κB. Knockdown of IκBη by siRNA suppressed the transcription of a subset of NF-κB–mediated proinflammatory cytokines in LPS-stimulated and poly (I:C)-transfected macrophages. These results indicate that IκBη regulates the NF-κB–mediated transcription of a wide variety of proinflammatory genes, playing a crucial role in the regulation of innate immune responses.

innate immunity | Toll-like receptor | macrophage | signal transduction | ankyrin repeat

The NF-κB/Rel family, consisting of p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RELB, and REL (c-Rel), play a central role in the regulation of inducible gene expression in various biological systems (1–3). In response to stimuli, a dimer of NF-κB proteins binds to a κB site in the promoter or enhancer of a target gene, resulting in the expression of various inflammatory genes. The signaling pathways of NF-κB have been characterized extensively in innate immune responses. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), recognize microbial components or viral nucleic acids and activate the NF-κB signal-transduction pathway to induce expression of a wide variety of inflammatory gene products, such as TNF-α, IL-6, and IFNs, playing a key role in innate immune responses (4).

NF-κB proteins are constitutively expressed in unstimulated cells, but their activities are strictly repressed by an inhibitor of NF-κB (IκB) protein (1, 5). Seven IκB proteins have been identified to date: IκBα, IκBβ, IκBε, IκBγ, BCL-3, IκBNS, and IκBζ. These proteins are characterized by multiple ankyrin repeats and interaction with an NF-κB subunit. In unstimulated cells, NF-κB forms an inert complex with a canonical IκB protein, such as IκBα or IκBβ, which masks the nuclear localization signal of NF-κB and sequesters the complex away from the nucleus. To activate NF-κB's transcriptional activity, the IκB protein needs to be released from the NF-κB/IκB complex (3). Upon stimulation, TLRs activate IκB kinase via a protein cascade, leading to phosphorylation of IκB bound to NF-κB. Phosphorylated IκB proteins were degraded by the proteasome system, releasing NF-κB from the inactive complex with canonical IκB proteins. Free NF-κB translocates into the nucleus and regulates transcription of target genes by binding to κB sites in their promoters or enhancers.

A wide variety of proinflammatory genes are regulated by NF-κB, and its regulatory mechanisms are also diverse because of cooperating with multiple regulatory factors. In addition to the canonical IκB proteins acting as inhibitors of NF-κB signal transduction in the cytoplasm, there are nuclear IκB proteins, such as BCL-3, IκBNS, and IκBζ, which are present in the nucleus and thought to regulate the transcriptional activity of NF-κB (5, 6). Although less expressed under normal conditions, these nuclear IκB proteins are highly inducible in response to stimuli. BCL-3 functions as either an activator or an inhibitor of NF-κB in a context-specific manner by regulating its transcriptional activity or its stability on DNA (7, 8). IκBNS inhibits IL-6 production by associating with DNA-bound p50 homodimers, preventing the binding of NF-κB dimers to the promoters (9, 10). IκBζ induced to express by stimuli binds to p50 on IL-6 and IL-12p40 promoters, and is supposed to activate transcription by remodeling the nucleosomes in targeted regions (11, 12). Therefore, the transcriptional activity of NF-κB is regulated not only by cytoplasmic IκBs, but also by nuclear IκBs, to strictly modulate the NF-κB–mediated expression of inflammatory genes. However, factors regulating NF-κB's activity in the nucleus still remain largely unknown.

In this article, we describe a unique molecule with ankyrin repeats that was found in a gene expression analysis of bone marrow-derived dendritic cells (BMDCs). Because it is structurally related to the IκB family, we have named the protein "IκBη" and further studied its expression and functions, especially in innate immune responses. We found that IκBη is predominantly located in the nucleus and interacts with the p50 subunit of NF-κB. We also showed that IκBη modulates the NF-κB–mediated transcriptional activity for the expression of a subset of proinflammatory genes. Based on these results, we propose that IκBη is a unique nuclear IκB protein playing a crucial role for regulating the expression of proinflammatory genes in innate immune responses.

Results

IκBη Is a Unique Member of the IκB Family. To find novel molecules involved in the regulation of immune responses, a microarray analysis was performed using mRNA from mouse BMDCs. Among many genes expressed in BMDCs, we were interested in one gene encoding ankyrin repeats, because ankyrin repeats are known to be important for interaction with NF-κB, a central regulator of inflammation (13). This gene was identical to the

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The authors declare no conflict of interest.

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[1]Present address: Immunology Program, Benaroya Research Institute at Virginia Mason, 1201 9th Avenue, Seattle, WA 98101.

[2]To whom correspondence should be addressed: E-mail: miyajima@iam.u-tokyo.ac.jp.

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Ankrd42 gene in databases, but its function had not been described. The protein encoded by the gene consists of 516 amino acid residues and has eight ankyrin repeats in the NH_2-terminal region and a coiled-coil domain in the COOH-terminal region (Fig. 1*A*). Its amino acid sequence, especially the ankyrin-repeats domain, is highly conserved in mouse, rat, and human, and closely related to that of the IκB family, well-known regulators of NF-κB signal transduction (Fig. 1*B* and Fig. S1*A*). Moreover, the protein was similar in function to IκB proteins as described below. Therefore, we have named it IκBη and further analyzed its expression and functions.

The tissue distribution of IκBη in adult mice was examined by Northern blotting (Fig. 1*B*). An ≈2.8-kb mRNA for IκBη was rather ubiquitously expressed in all of the tissues examined and highly expressed in the brain, lung, testis, and ovary. As NF-κB mediates a central signaling pathway in innate immune responses and IκB family proteins are major components of NF-κB signal transduction, we examined the expression of IκBη in antigen-presenting cells by RT-PCR assay and revealed that IκBη was expressed not only in dendritic cells but also in macrophages in the spleen (Fig. S1*B*). IκBη was also expressed in T cells and B cells (Fig. S1*B*). These results indicate that IκBη is ubiquitously expressed in various tissues and blood cells, including antigen-presenting cells.

Expression and Subcellular Distribution of IκBη. Because the expression of IκB proteins is known to be highly inducible, we examined whether the expression of IκBη was regulated by TLR signals by real-time RT-PCR (Fig. 2*A*). Expression of *Ikbh* mRNA in the macrophage cell line Raw264.7 was constitutive and only slightly up-regulated by LPS. In addition to LPS, zymosan, poly (I:C) and CpG DNA, ligands for TLR2, TLR3, and TLR9, respectively, also only marginally increased the expression of IκBη in Raw264.7 cells (Fig. 2*A*). The induction was

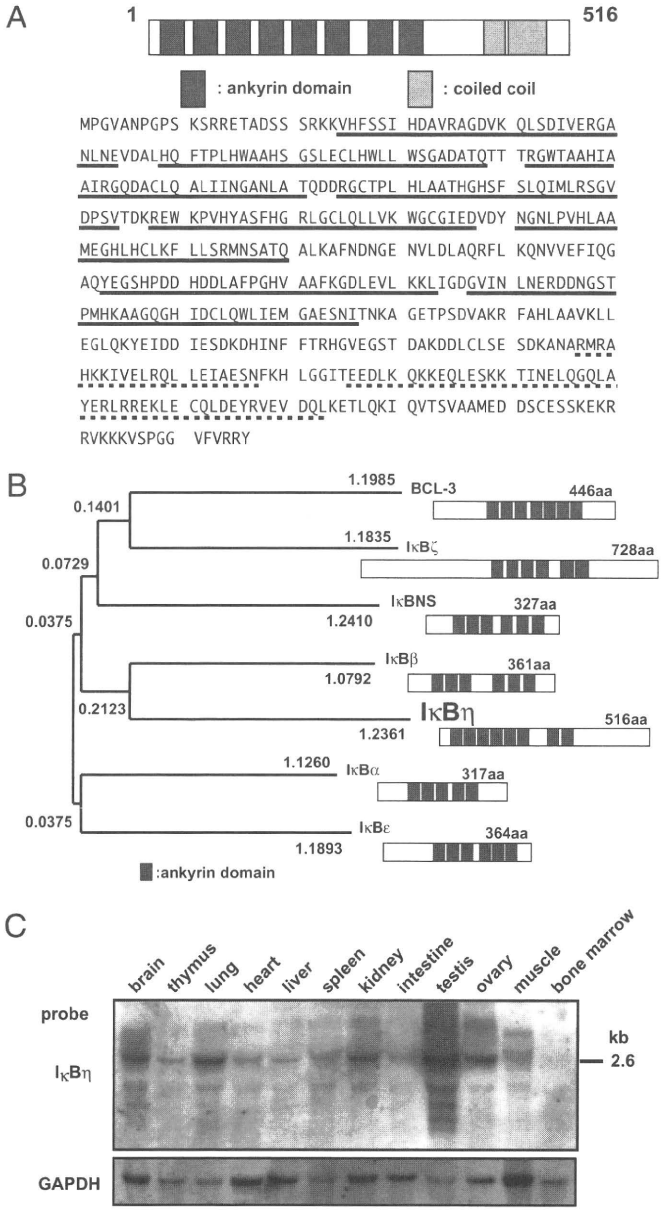

Fig. 1. Structure and tissue distribution of mouse IκBη. (*A*) The amino acid sequence and a structural diagram of IκBη. The ankyrin repeats and coiled-coil domains are indicated by blocks and boxes, respectively. Black underlines indicate ankyrin domains and the dotted underline indicates the putative coiled-coil region. (*B*) A phylogenic tree of mouse IκB family proteins, obtained using the Neighbor-Joining method. Diagrams of each IκB protein and ankyrin domains are shown on the right. (*C*) Northern blot of mouse tissues probed with mouse IκBη or the GAPDH control probe. Each lane contains 20 μg of total RNA. The position of the 2.6 kb marker is indicated.

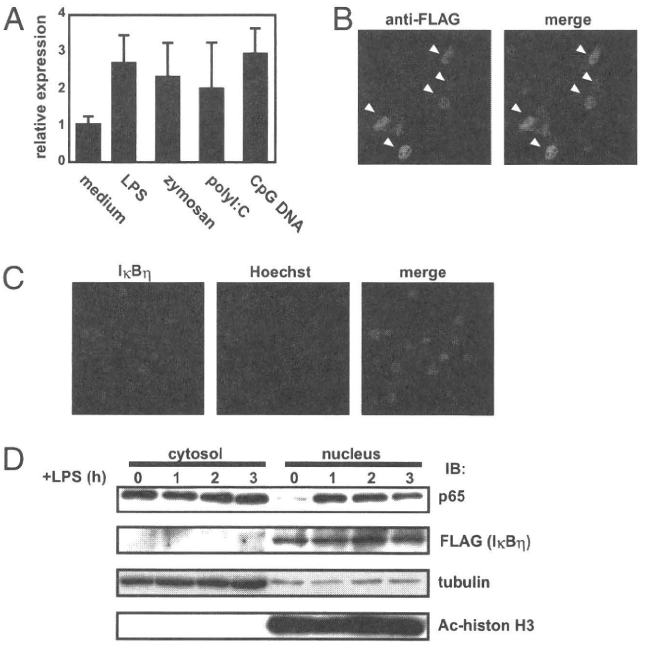

Fig. 2. Expression and subcellular distribution of IκBη. (*A*) Raw264.7 cells were treated with medium, LPS (100 ng/mL), zymosan (1 mg/mL), poly (I:C) (25 μg/mL), and CpG DNA (1 μM) for 90 min and the expression of *Ikbh* and *Hprt* mRNA was measured by real-time RT-PCR. The expression of IκBη was normalized to that of the housekeeping gene *Hprt*. The data are shown as the relative expression of *Ikbh* compared with untreated cells. (*B*) NIH 3T3 cells were transfected with FLAG-tagged IκBη for 24 h. Cells were stained with anti-FLAG Ab (green) as well as Hoechst dye (blue), and analyzed by microscopy. Arrowheads indicate transfected cells. (*C*) NIH 3T3 cells were stained with anti-IκBη Ab (green) as well as Hoechst dye (blue), and analyzed by microscopy. (*D*) Raw264.7 cells were transiently transfected with FLAG-tagged IκBη and then stimulated with LPS. Cytoplasmic and nuclear fractions were isolated and subjected to Western blotting using anti-IκBα, anti-p65, anti-FLAG Abs. Tubulin and acetyl-histone H3 were used as a cytoplasmic and nuclear marker, respectively. Data shown are representative of two or three experiments.

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.

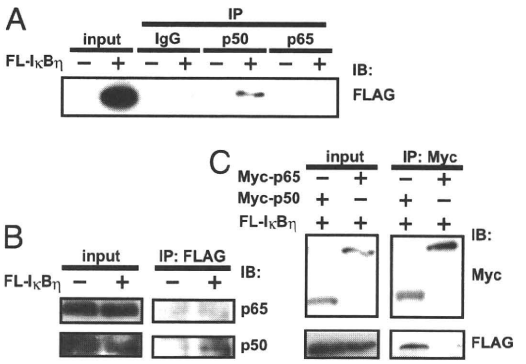

Fig. 3. Interaction of IκBη with the p50 subunit of NF-κB. (*A*) NIH 3T3 cells were transfected with control GFP or FLAG-tagged IκBη for 24 h and proteins were immunoprecipitated (IP) with anti-p50 Ab, anti-p65 Ab, or control rabbit IgG Ab. The immunoprecipitates were immunoblotted with anti-FLAG Ab. (*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

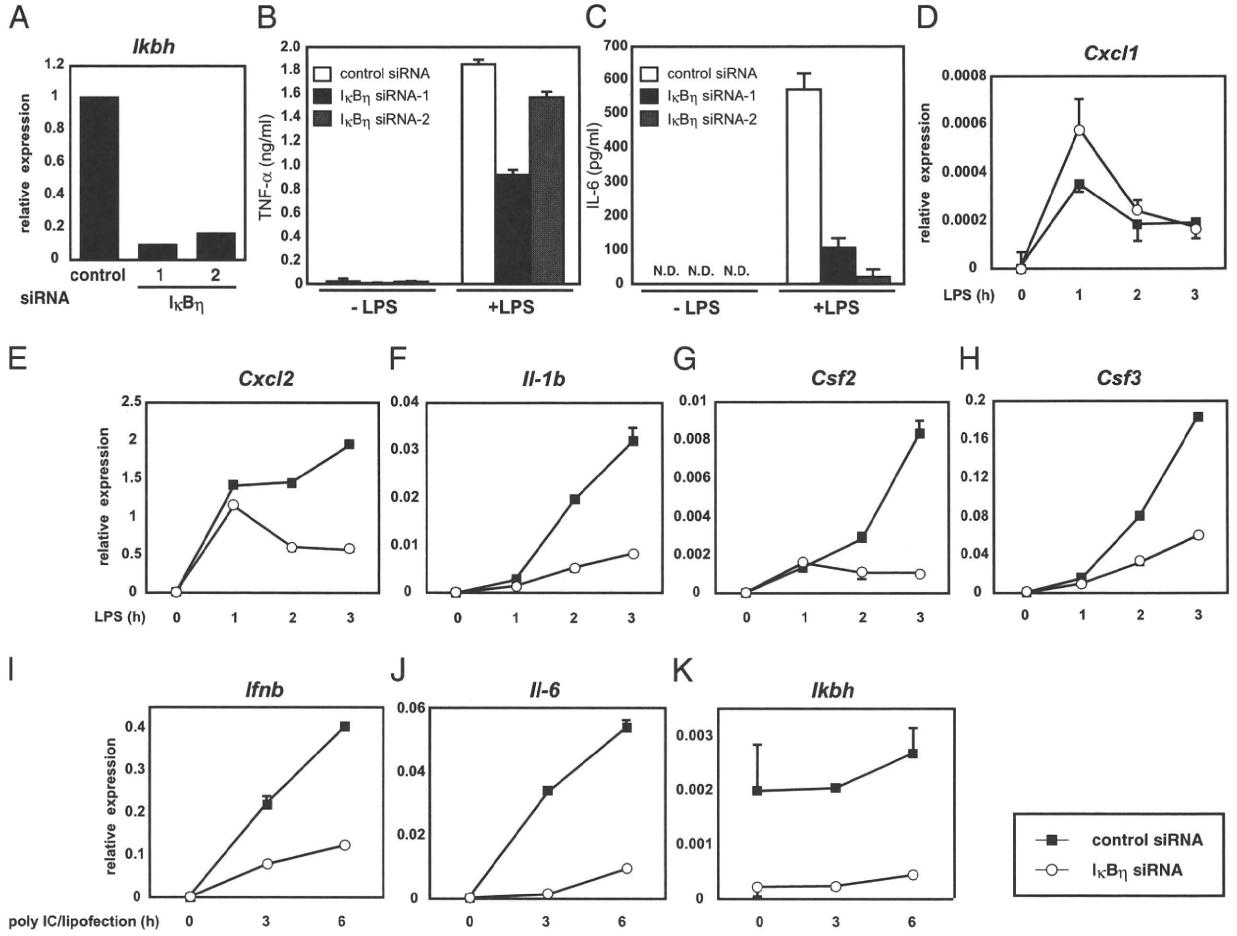

Fig. 4. IκBη regulates NF-κB-activated proinflammatory gene expressions in innate immune responses. (*A*) Raw264.7 cells were transfected with control or IκBη siRNA (1 or 2). Expression of IκBη mRNA was determined by real-time RT-PCR. The expression levels were normalized to that of the housekeeping 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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Hiroyuki Yanai[1,2,*], Koji Nakamura[1], Susumu Hijioka[3], Akira Kamei[3], Takaaki Ikari[3], Yuichi Ishikawa[4], Eiji Shinozaki[2], Nobuyuki Mizunuma[2], Kiyohiko Hatake[2] and Atsushi Miyajima[5]

[1]LivTech Inc., Teikyo University Biotechnology Research Centre 1F, 907 Nogawa, Miyamae, Kawasaki, Kanagawa 216-0001; [2]Department of Medical Oncology; [3]Department of Gastroenterology; [4]Department of Pathology, Cancer Institute Hospital, Japanese Foundation for Cancer Research; and [5]Laboratory of Cell Growth and Differentiation, Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan

*Hiroyuki Yanai, Livtech Inc., Teikyo University Biotechnology Research Centre 1F, 907 Nogawa, Miyamae, Kawasaki, Kanagawa, 216-0001, Japan. Tel: +81 44 751 3084, Fax: +81 44 751 3381, E-mail: yanai@livtech.co.jp

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 μg/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).

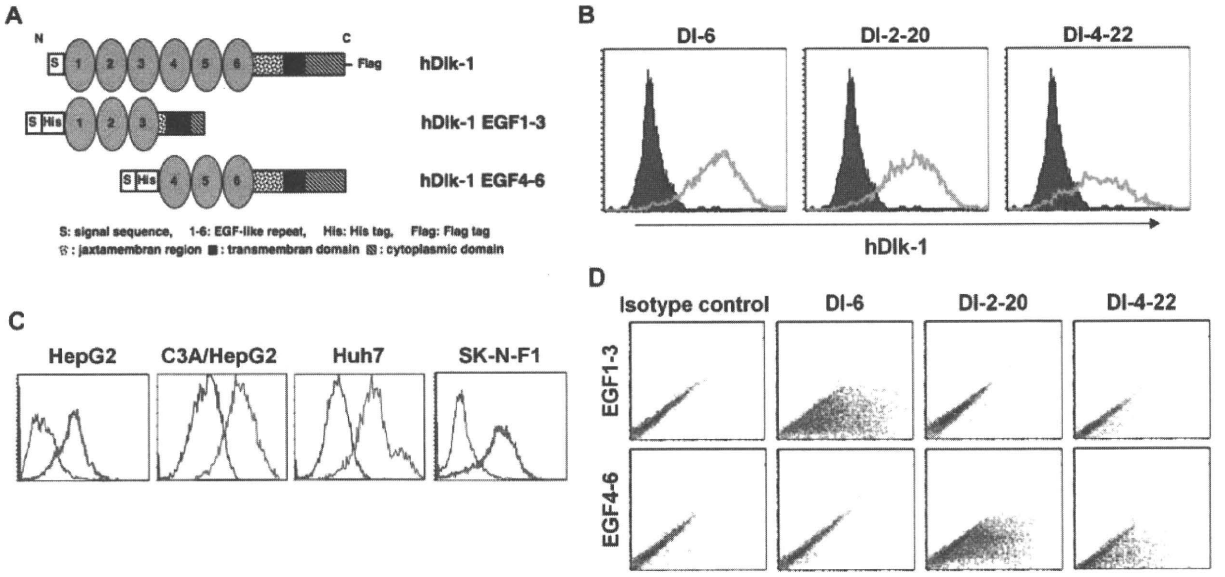

Fig. 1 Characterization of anti-hDlk-1 monoclonal antibodies by FACS analysis. (A) Schematic representation of various hDlk-1 constructs used in this study. (B) Three monoclonal antibodies used in this study specifically recognize hDlk-1 expressing cells. HEK-293 cells stably-expressing hDlk-1 were harvested, 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.

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

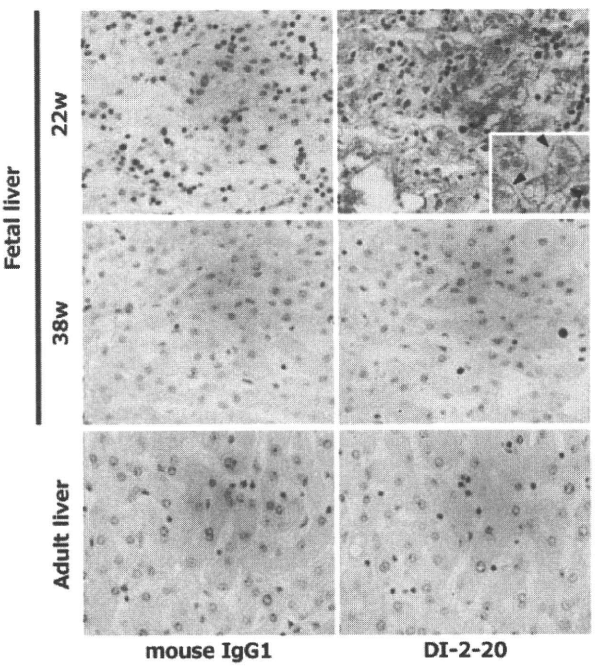

Fig. 2 Immunohistochemical analysis of hDlk-1 in foetal and adult liver. Each specimen was stained with anti-hDlk-1 mAb DI-2-20 (right panels). Foetal liver tissue at 22w (upper) shows membrane (arrow heads) and cytoplasmic staining with DI-2-20 monoclonal antibody. hDlk-1 staining was not observed in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

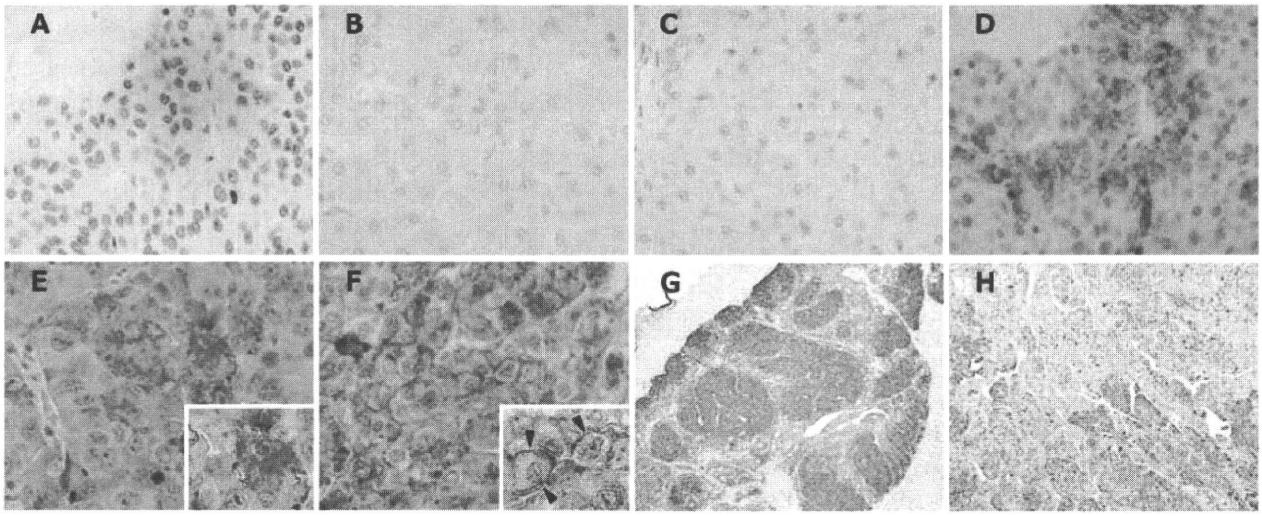

Fig. 3 hDlk-1 expression was observed only in hepatocellular carcinoma. Immunohistochemical staining with anti-hDlk-1 monoclonal antibody DI-2-20 was performed using tissue microarrays of liver tumour (388 malignant and 19 benign), nodular cirrhosis (40), viral hepatitis (11) and normal liver (26). The 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 χ^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				
40>	29	22	43.10%	
40–49	82	29	26.10%	
50–60	99	17	14.70%	
>60	93	11	10.60%	P<0.01
AFP				
−	179	25	12.30%	
+	26	25	49.00%	P<0.01
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. 4G 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

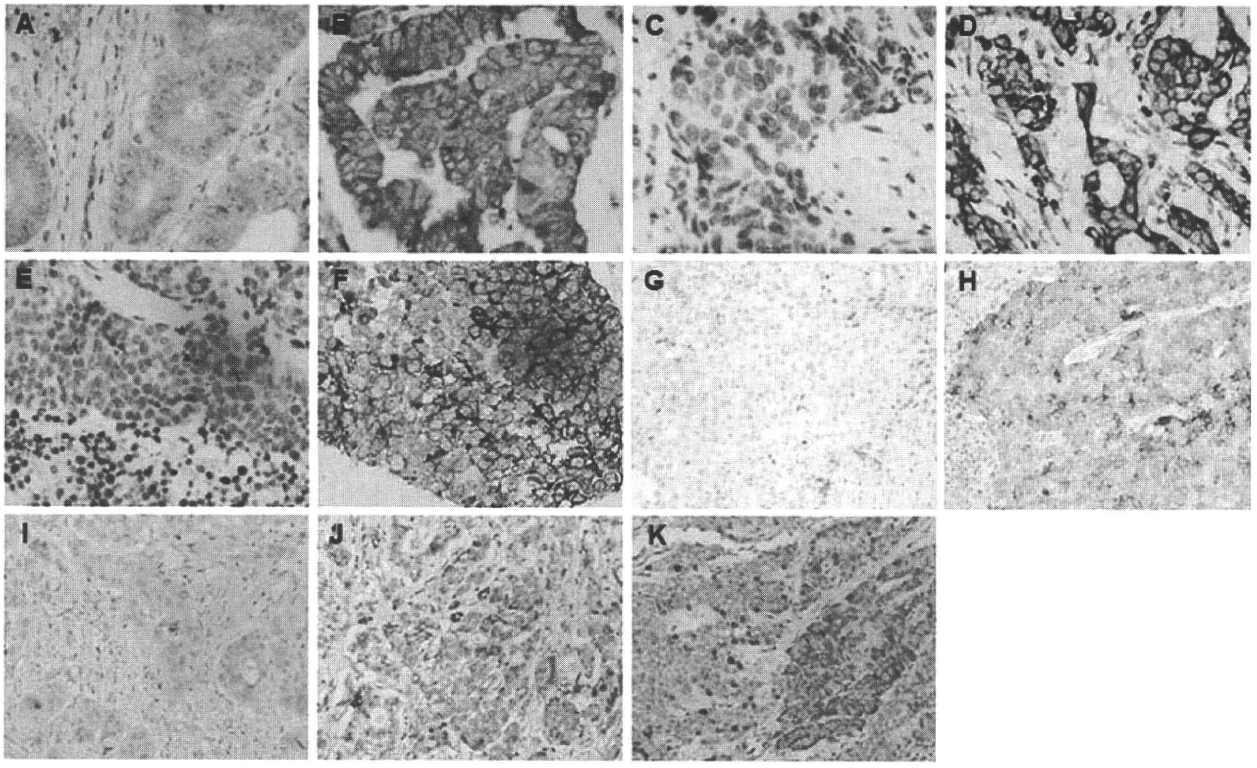

Fig. 4 hDlk-1 expression in various tumours. hDlk-1 expression in various tumours were examined by immunohistochemistry using anti-hDlk-1 mAb, DI-2-20. (A and B) Colon adenocarcinoma, (C and D) breast carcinoma, (E and F) small cell lung carcinoma, (G and H) AFP-producing gastric cancer, (I and J) pancreatic 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., Halász, J., Bisgaard, H.C., Paku, S., Turányi, 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. Baladrón, V., Ruiz-Hidalgo, M.J., Nueda, M.L., Díaz-Guerra, M.J., García-Ramírez, 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