の分化誘導系を使って，肝細胞分化の分子機構の解析が可能となり，STAT3がTATなどの肝酵素の発現促進やD1 cyclinの発現抑制，K-Rasによる細胞接着構造の形成など細胞内シグナル分子の機能解析が進んだ7~9)．

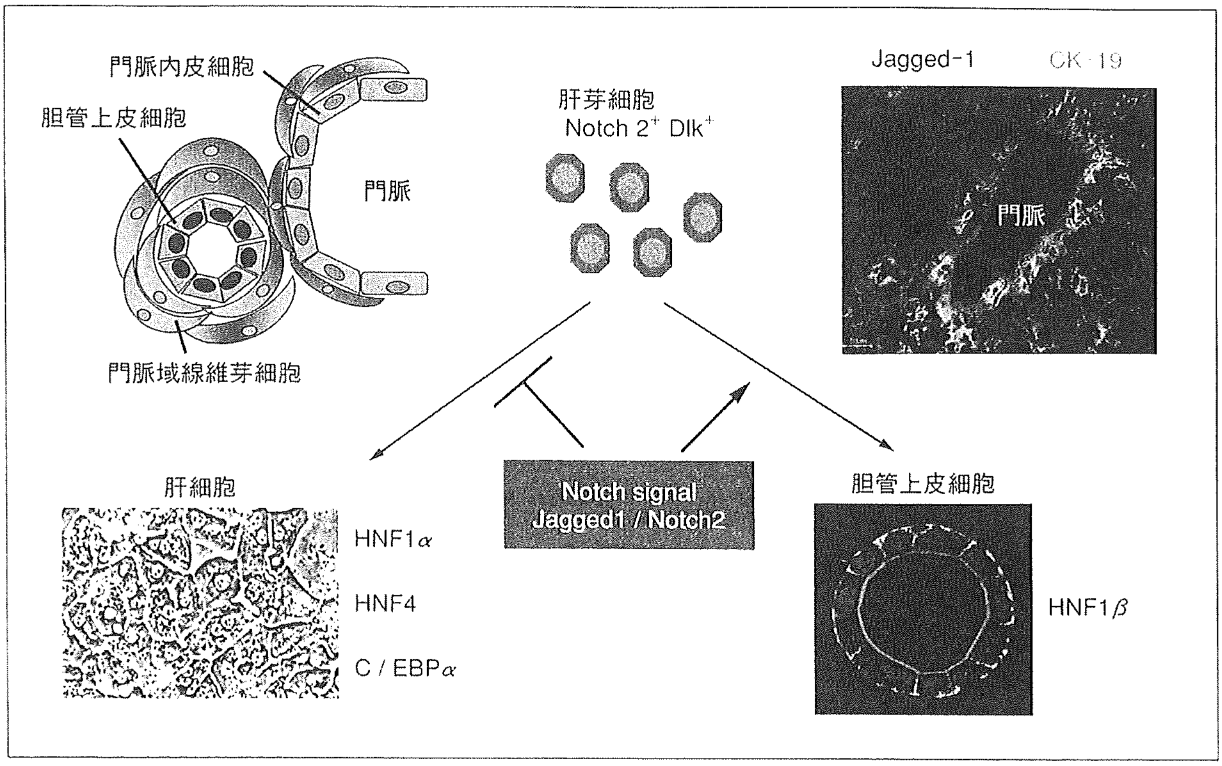
肝細胞の多くの代謝関連酵素の発現は出生時に劇的に誘導される．C／EBPα欠損マウスは出生後に低血糖と高アンモニア血症を伴って死亡することから，これらに関与する酵素発現にC／EBPαが必須である10)．一方，C／EBPαは胎生肝臓にも発現しており，C／EBPαの発現のみでは代謝酵素の発現は説明されない．我々は，糖新生に必須のPEPCKの発現はC／EBPαとFoxo1の協調的な作用を必要とすることが明らかにした11)．Foxo1は出生直前に発現誘導される．また，Foxo1の活性はインスリンにより負の制御を受けているが，インスリンレベルは出生前に高く，出生により激減することから，Foxo1はこのインスリンの変化を感知して糖新生を行うことが示唆される．

アンモニアの分解に関与するCPS1の発現もC／EBPα依存的であるが，胎児肝臓にはYB-1が発現しており，それがC／EBPαによるCPS1の発現を制御していることも明らかになった．成体肝臓ではYB-1の発現は抑制されているが，肝障害によりYB-1発現が誘導され，それに伴いCPS1が低下して高アンモニア血症になる12)．このように，複数の転写因子の共同作用の様子が徐々に明らかにされてきた．

胆管形成

肝芽細胞は増殖して肝細胞と胆管上皮細胞へと分化するが，胆管は門脈周囲にのみ形成される．Allagile syndromeは肝内胆管の形成不全を伴い，その原因遺伝子がJagged1であることから，胆管形成へのNotchシグナルの関与が示唆されていた13)．肝芽細胞にはNotch2が発現しており，Jagged1は門脈周囲に発現すること，分離したDlk陽性細胞にNotchの活性化型であるNotch細胞内ドメインを強制発現すると，胆管分化が促進され肝細胞分化が抑制された14)．一方，門脈域のJagged1陽性細胞はp75NTR陽性の間葉系細胞である15)．したがって，Notch2を発現するDlk陽性の肝芽細胞がJagged1を発

図１ 肝幹細胞の分化



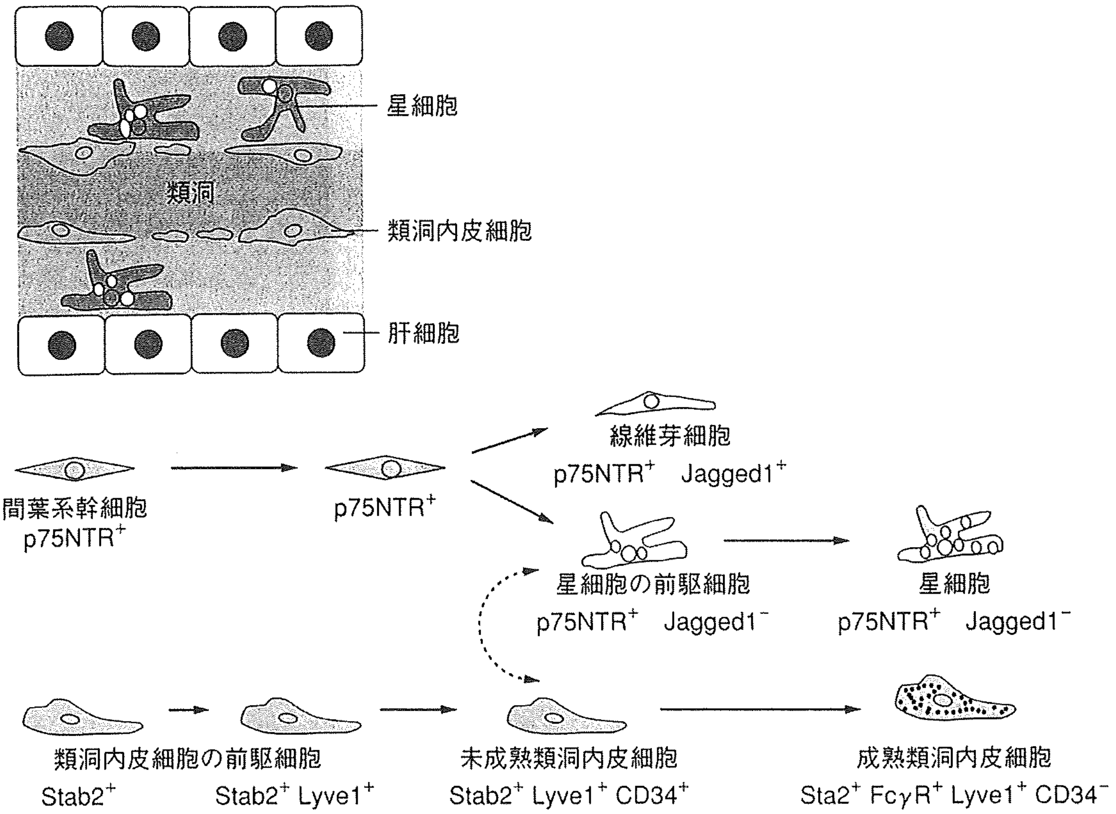
胎児肝臓の肝芽細胞は Dlk 陽性で Notch2 を発現している．門脈域の p75NGF 陽性の線維芽細胞は Jagged1 を発現している．肝芽細胞で Notch を活性化すると肝細胞への分化が抑制され胆管上皮細胞への分化が促進される．
HNF：肝細胞核結合因子，C / EBPα：CCAAT / エンハンサー結合タンパク質 α

現する門脈域の線維芽細胞の作用により，肝細胞への分化が抑制されて胆管上皮への分化が誘導されると考えられる（図１）．

類洞壁細胞

肝類洞は肝臓に特有の毛細血管系であり，類洞壁を構成する内皮細胞は基底膜を持たず有窓構造（fenestrae）を有し，遺伝子発現のプロファイルも他の内皮細胞とは異なるユニークな内皮細胞である．この細胞には，ヒアルロン酸受容体である Lyve-1 と Stabilin-2 (Stab2) が発現するが，E9.5 日の肝芽でもすでにこれらの発現が認められる．E14 日の肝類洞内皮細胞は CD34 陽性 FcγR 陰性であるが，成体では CD34 陰性 FcγR 陽性となる．類洞内皮細胞はリンパ管や血管内皮細胞などに比べて強い endocytosis 活性を有する（図２）[16]．

図2　類洞の構造と類洞壁細胞の分化



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

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

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

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

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

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

おわりに

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

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

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

文　献

1) Zaret K, et al: Generation and regeneration of cells of the liver and pancreas. Science 322: 1490-1494, 2008.

2) Tanimizu N M, et al: Isolation of hepatoblasts based on the expression of Dlk/Pref-1. J Cell Sci 116: 1775-1786, 2003.

3) Moon Y S, et al: Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. Mol Cell Biol 15: 5585-5592, 2002.

4) Tanimizu N, et al: Long-term culture of hepatic progenitors derived from mouse Dlk+ hepatoblasts. J Cell Sci 117: 6425-6434, 2004.

5) Kamiya A, et al: Fetal liver development requires a paracrine action of oncostatin M through gp130. EMBO J 18: 2127-2136, 1999.

6) Kamiya A, et al: Maturation of fetal hepatocytes in vitro by extracellular matrices and Oncostatin M; Induction of tryptophan oxygenase. Hepatology 35: 1351-1359, 2002.

7) Ito Y, et al: Retroviral gene transfer of signaling molecules into murine fetal hepatocytes defines distinct roles for the STAT3 and Ras pathways during hepatic development. Hepatology 32: 1370-1376, 2000

8) Matsui T, et al: STAT3 down-regulates the expression of cyclin D during liver development. J Biol Chem 277: 36167-36173, 2002.

9) Matsui T, et al: K-Ras mediates cytokine-induced formation of E-cadherin-based adherens junctions during liver development. EMBO J 21: 1021-1030, 2002.

10) Darlington G J, et al: C/EBP alpha: a critical regulator of genes governing integrative metabolic processes. Curr Opin Genet Dev 5: 565-570, 1995.

11) Sekine K, et al: Foxo1 links insulin signaling to C/EBPα and regulates gluconeogenesis during liver development. EMBO J 26: 3607-3615, 2007.

12) Chen Y-R, et al: YB-1 downregulates expression of carbamoyl phosphate synthetase 1 by suppressing C/EBPα function, leading to hyperammonemia. Gastroenterology 2009.

13) Li L, et al: Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat Genet 3: 243-251, 1997.

14) Tanimizu N, et al: Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. J Cell Sci 117: 3165-3173, 2004.

15) Suzuki K, et al: The p75 neurotrophin receptor marks precursors of hepatic

stellate cells and portal fibroblasts in mouse fetal liver. Gastroenterology 135: 270–281, 2008.

16) Nonaka H, et al: Development of murine hepatic sinusoidal endothelial cells characterized by the expression of hyaluronan receptors. Dev Dyn 236: 2258–2267, 2007.

Liver Development and Differentiation

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Hematopoiesis-dependent expression of CD44 in murine hepatic progenitor cells

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ABSTRACT

The fetal liver serves as the predominant hematopoietic organ until birth. However, the mechanisms underlying this link between hematopoiesis and hepatogenesis are unclear. Previously, we reported the isolation of a monoclonal antibody (anti-Liv8) that specifically recognizes an antigen (Liv8) present in murine fetal livers at embryonic day 11.5 (E11.5). Liv8 is a cell surface molecule expressed by hematopoietic cells in both fetal liver and adult mouse bone marrow. Here, we report that Liv8 is also transiently expressed by hepatoblasts at E11.5. Using protein purification and mass spectrometry, we have identified Liv8 as the CD44 protein. Interestingly, the expression of Liv8/CD44 in fetal liver was completely lost in *AML1*$^{-/-}$ murine embryos, which lack definitive hematopoiesis. These results show that hepatoblasts change from Liv8/CD44-negative to Liv8/CD44-positive status in a hematopoiesis-dependent manner by E11.5, and indicate that Liv8/CD44 expression is an important link between hematopoiesis and hepatogenesis during fetal liver development.

Fetal liver is known to serve as the predominant hematopoietic organ during its own proliferation and differentiation until birth [1]. Hepatogenesis and hematopoiesis have close interactions as follow. Hepatoblasts, which are hepatic progenitor cells, are thought to support hematopoiesis because hepatocyte-like cell lines were reported to be able to support hematopoiesis [2,3]. On the other hand CD45-positive (CD45^{+}) hematopoietic cells in the fetal liver produce an Interleukin-6 (IL-6) family cytokine, Oncostatin M (OSM), to promote the development of hepatocytes in the middle to late liver development [4,5]. However, the molecules linking hepatogenesis and hematopoiesis during early fetal liver development remains unknown.

The process of embryonic liver development can be divided into several distinct stages [6]. The liver primordium proliferates and invades the septum transversum mesenchyme to give rise to the hepatic codes and buds at E9.5. Hepatic cells at this stage, called hepatoblasts, possess the potential to differentiate into both parenchymal hepatocytes and bile duct epithelial cells. At around E10.5, hematopoietic stem cells originating from aorta–gonad–mesonephros (AGM) region colonize the fetal liver and expand their mass and lineage diversity, such as erythrocytes [1,7]. Hepatoblasts participate in creating the hematopoietic microenvironment in concert with other stromal cells to promote embryonic hematopoiesis [2,3].

We have prepared several monoclonal antibodies specifically recognizing murine fetal livers because molecular markers and tools were needed to understand the mechanisms of early liver development [8,9]. One of the antibodies, called anti-Liv2,

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recognized hepatoblasts specifically at E9.5–E12.5. We could analyzed hepatoblast proliferation in knockout mice using anti-Liv2 [9,10]. Furthermore, we have reported another antibody, anti-Liv8, that recognize both hematopoietic progenitor cells in fetal liver at E11.5 and $CD45^+$ hematopoietic cells in adult bone marrow [11,12]. However, its molecular identification of the Liv8-antigen (Liv8) and its expression in embryonic development were remained unknown.

In this report, we show a molecular identification of Liv8 as CD44. Our finding suggest that Liv8/CD44 links hematopoiesis and hepatogenesis by its the adhesive activity and signaling role during fetal liver development.

Materials and methods

Mice. C57BL/6J mice were purchased from CLEA Japan. *AML1* mutant mice were generated as described previously [13].

Antibodies. Anti-Liv2 and anti-Liv8 antibodies were prepared and purified as described [9]. Anti-Liv8 antibody was biotinylated by using EZ link Sulfo-NHS-Biotin (Pierce) and anti-Liv2 antibody was labeled with Alexa Fluor 488 by using Zenon labeling kit (Invitrogen). Fluorescein isothiocyanate (FITC)-conjugated and unconjugated anti-CD44 (IM7 and KM114), FITC-conjugated anti-CD71 (C2) and phycoerythrin (PE)-conjugated anti-TER-119 antibodies were purchased from Becton Dickinson Biosciences, anti-Myc (9E10) and anti-FLAG (M2) antibodies were from Sigma, anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 6C5) antibody was from CHEMICON.

Immunohistochemistry. Paraffin-embedded and frozen sections were immunostained according to previously described protocols [9].

Cell culture and transfection. LO cells were established as described previously [14], and maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 15% fetal calf serum (FCS), 10 ng/mL mouse OSM (Sigma) and antibiotics. cDNA encoding CD44 tagged with FLAG at C-terminal (FLAG-CD44) were cloned into mammalian expression vector pCMV5. For gene expression analysis, 293T cells were plated and transfected 1 day later with 5 μg of plasmid DNA using LipofectAMINE 2000 (Invitrogen).

Immunoblotting. Fetal liver (E11.5), LO and COS-7 cells were washed and homogenized in phosphate-buffered saline (PBS). The homogenates were centrifuged at 900*g* for 10 min at 4 °C, and the supernatants were centrifuged at 100,000*g* for 30 min at 4 °C. The resulting membrane pellets were used as membrane fractions. For the deglycosylation of membrane fraction, samples were incubated with PNGase F (PROzyme) or neuraminidase (nacalai tesque) according to the manufacturer's instructions. Immunoprecipitation and immunoblotting were performed as described [15].

Purification of Liv8-antigen and mass spectrometry. LO membrane fraction was lysed in 50 mM Tris–HCl (pH 7.5), 1% Lubrol PX (lysis buffer), and centrifuged at 100,000*g* at 4 °C for 1 h. The supernatant was applied on WGA agarose column (Seikagaku Corporation) equilibrated with lysis buffer. The column was washed with 1 mM phosphate buffer (pH 7.4), 1 M NaCl, 0.1% sodium-cholate (wash buffer), and the protein was eluted with wash buffer containing 250 mM N-acetyl-D-glucosamine (GlcNAc). The GlcNAc eluant was then applied on Phenyl-Sepharose column (GE healthcare) equilibrated with wash buffer, and the protein was eluted with a liner gradient of 1000–0 mM NaCl and 0.1–2% of sodium-cholate. The Liv8-positive fractions were adjusted to 1 mM $CaCl_2$, then applied on the hydroxyapatite Bio-Gel HTP Gel column (Bio-Rad) equilibrated with 1 mM phosphate buffer, 0.3 mM $CaCl_2$, 0.1 % Lubrol PX. The protein was eluted with a liner gradient of 1–400 mM phosphate containing 0.1% Lubrol PX and 0.1 mM EDTA. The Liv8-positive fractions were diluted with 50 mM Tris–HCl (pH 7.5), 0.1% Lubrol PX (dilution buffer) and applied on Mono Q HR5/5 column (GE healthcare) equilibrated with dilution buffer. The protein was eluted with a liner gradient of 0–1 M NaCl in dilution buffer. Purified Liv8-antigen was concentrated with VIVASPIN (VIVASCIENCE) and separated by SDS-PAGE. The proteins were visualized with Silver Stain II Kit Wako or negative gel stain MS kit according the manufacturer's instructions (Wako). The appropriate position was cut out and washed with 5% acetate, 50% methanol, and dehydrated with 66% acetonitrile, 17 mM NH_4HCO_3. The protein was reduced by 10 mM dithiothreitol, alkylated by 55 mM iodoacetamide, and digested by trypsin. The peptide solution was placed on the target tip with α-cyano-4-hydroxy cinnamic acid as the matrix. The spectra of the peptides were obtained using Ultraflex mass spectrometer (BRUCKER) and Voyager DE-STR (Applied Biosystems).

Fluorescence-activated cell sorter (FACS) analysis. Freshly isolated fetal livers were incubated with Liver Perfusion Medium and Liver Digestion Medium (Invitrogen) at 37 °C. The cells were dissociated by pipetting, washed with PBS, and reacted with fluorescein-labeled antibodies according to previously described protocol [11]. The labeled cells were analyzed using FACSCalibur (Becton Dickinson Biosciences).

Results

Expression of Liv8 in murine fetal liver

As almost all cells were $Liv8^+$ in fetal liver at E11.5 (Fig. 1A), we first examined the expression of Liv8 in hepatoblasts, and found that Liv8 is expressed in $Liv2^+$ hepatoblasts in fetal liver at E11.5 (Fig. 1B; arrowheads). Next, we examined the expression of Liv8 in fetal livers during E9.5 to E13.5. Interestingly, Liv8 was not expressed in hepatoblasts at E9.5 (Fig. 1C, dotted line). The ratios of $Liv8^+$ cells in fetal livers transiently increased and reached its maximum at E11.5, and then decreased at E12.5. Thus, hepatoblasts were $Liv8^-$ at E9.5 and became $Liv8^+$ at E11.5.

Previously, we reported that hematopoietic progenitor cells in fetal liver are $Liv8^+$ at E11.5 [11]. So, we next investigated the expression of Liv8 in AGM region, in which adult-type definitive hematopoiesis begins (Supplementary Fig. S1). Endothelial cells in AGM region at E9.5 were $Liv8^+$, and putative hematopoietic cells protruding from dorsal aorta at E11.5 were also $Liv8^+$. These results indicate that hematopoietic progenitor cells are $Liv8^+$ and hepatoblasts change from $Liv8^-$ at E9.5 to $Liv8^+$ at E11.5 in developing fetal livers.

Identification of Liv8 as CD44

To identify Liv8, we screened $Liv8^+$ cell line and found an endothelial-like cell line, LO cell, established from AGM region [14]. A band of molecular weight of 90-kDa recognized by anti-Liv8 antibody was detected in murine fetal liver and LO cells but not COS-7 cells (Fig. 2A). The band was disappeared by incubation with PNGase F that cleaves asparagine-linked oligosaccharides from glycoprotein, and was shifted to low molecular weight side by incubation with sialidase (Supplementary Fig. S2). These results indicate that Liv8-antigen is a 90-kDa glycoprotein, so we screened Liv8-antigen binding lectins and found Liv8-antigen bind to wheat germ agglutinin (WGA), a lectin that binds to hybrid type asparagines-linked oligosaccharides (data not shown). We purified Liv8-antigen from LO cells by various columns including WGA column, using anti-Liv8 blot as an index (see Materials and methods). Purified Liv8 was subjected to mass analysis. The amino acid sequence of

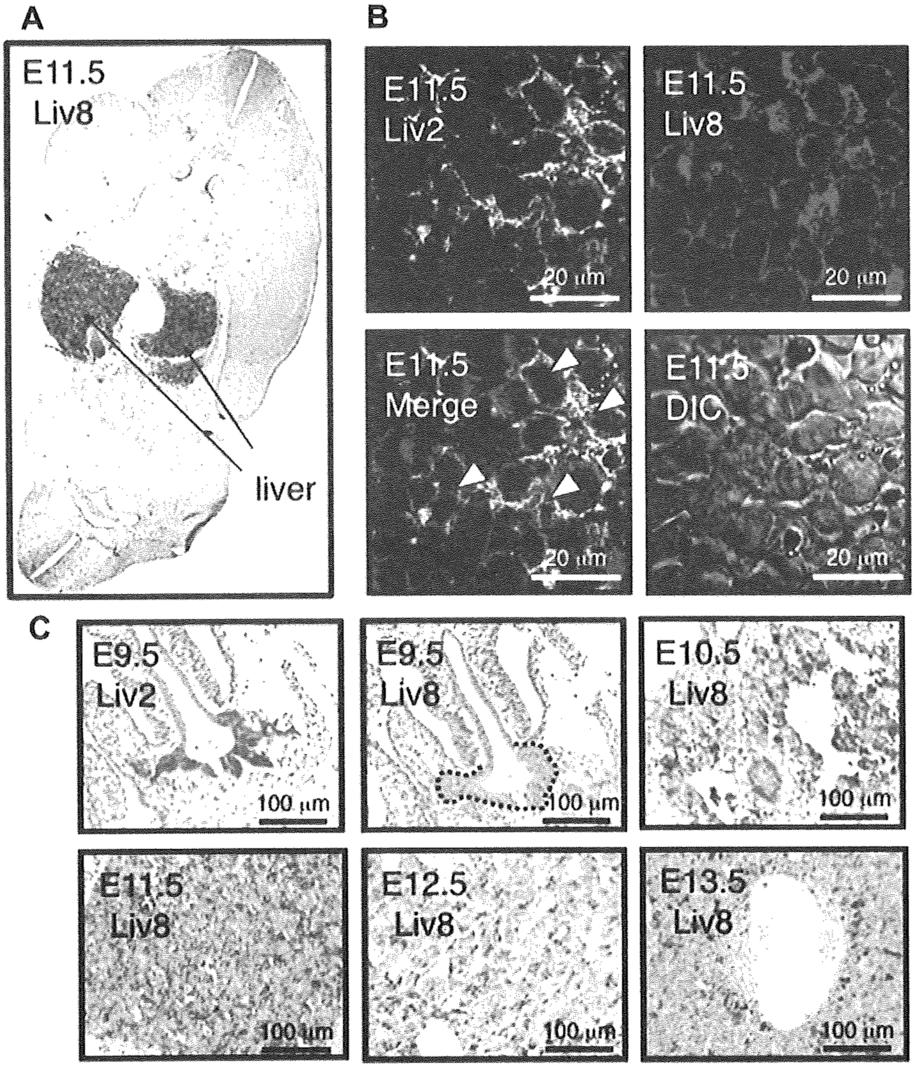

Fig. 1. Expression of Liv8 in murine fetal liver. (A) A transverse paraffin-section of murine embryo at E11.5 was stained with an anti-Liv8 antibody. (B) Transverse frozen section of murine embryonic liver at E11.5 was stained with anti-Liv2 (green) and anti-Liv8 (red) antibodies. Differential interference contrast (DIC) photograph image is also shown. Arrowheads indicate Liv8 expressing hepatoblasts. Scale bars indicate 20 μm. (C) Transverse paraffin-sections of murine embryonic liver at E9.5–13.5 were stained with anti-Liv8 or anti-Liv2 antibodies. Positive cells exhibit a brown precipitate and dotted line indicates hepatic bud at E9.5. Nuclei were counterstained with hematoxylin (purple). Scale bars indicate 100 μm.

spectrum observed at m/z 1363.7 from Liv8 was 'YGFIEGNVVIPR' which corresponded to the fragment from CD44.

To examine whether anti-Liv8 antibody recognize CD44, FLAG-CD44 was expressed in 293T cells and analyzed by anti-Liv8 blot (Fig. 2B). Anti-Liv8 and anti-FLAG antibodies recognized FLAG-CD44. Anti-Liv8 antibody also recognized immunoprecipitant of endogenous CD44 from LO cells (Fig. 2C). Finally, E12.5 fetal liver cells were analyzed by FACS using anti-Liv8 and anti-CD44 antibodies, and signals by each antibody were completely coincident (Fig. 2D). These results clearly show that Liv8 is identical to CD44.

Co-localization of Liv8/CD44 and hyaluronic acid in fetal liver but not AGM region at E11.5

CD44 was reported to interact with hyaluronic acid (HA) which is a major component of extra-cellular matrix (ECM) and function in cell adhesion [16]. To examine the interaction between Liv8/CD44 and HA in AGM region and fetal liver, we performed immunohistologic examination of E11.5 embryos using the biotinylated hyaluronic acid binding protein (HABP) and anti-Liv8. Liv8/CD44 and HA did not co-localized in AGM region at E11.5 (Fig. 3A). On the other hand, HA was well co-localized with Liv8/CD44 in fetal liver (Fig. 3B). This result suggests that Liv8/CD44 plays a pivotal role in the adhesion between the hematopoietic cells and hepatoblasts in fetal livers at E11.5.

If Liv8/CD44 functions in hematopoietic cells to adhere with fetal liver, the expression is expected to decrease with their maturation to leave fetal liver. So, we examined changes in Liv8/CD44 expression of hematopoietic cells accompanied with their differentiation to erythroid (Fig. 3C). Erythroid differentiation can be monitored step by step and quantitatively by FACS; this analysis distinguishes CD71 (transferrin receptor) and TER119 (erythroid lineage marker) double-stained erythroblasts into different stages of differentiation [17]. We triple-stained E15.5 fetal liver cells with anti-Liv8, CD71, TER119 antibodies and divided them into five populations by their characteristic staining patterns with anti-CD71 and anti-TER119 antibodies. These cells differentiate from R1 to R5 i.e., $\mathrm{CD71^{high}TER119^{low}}$, $\mathrm{CD71^{high}TER119^{meddle}}$, $\mathrm{CD71^{high}TER119^{high}}$, $\mathrm{CD71^{meddle}TER119^{high}}$, and $\mathrm{CD71^{low}TER119^{high}}$. Quan-

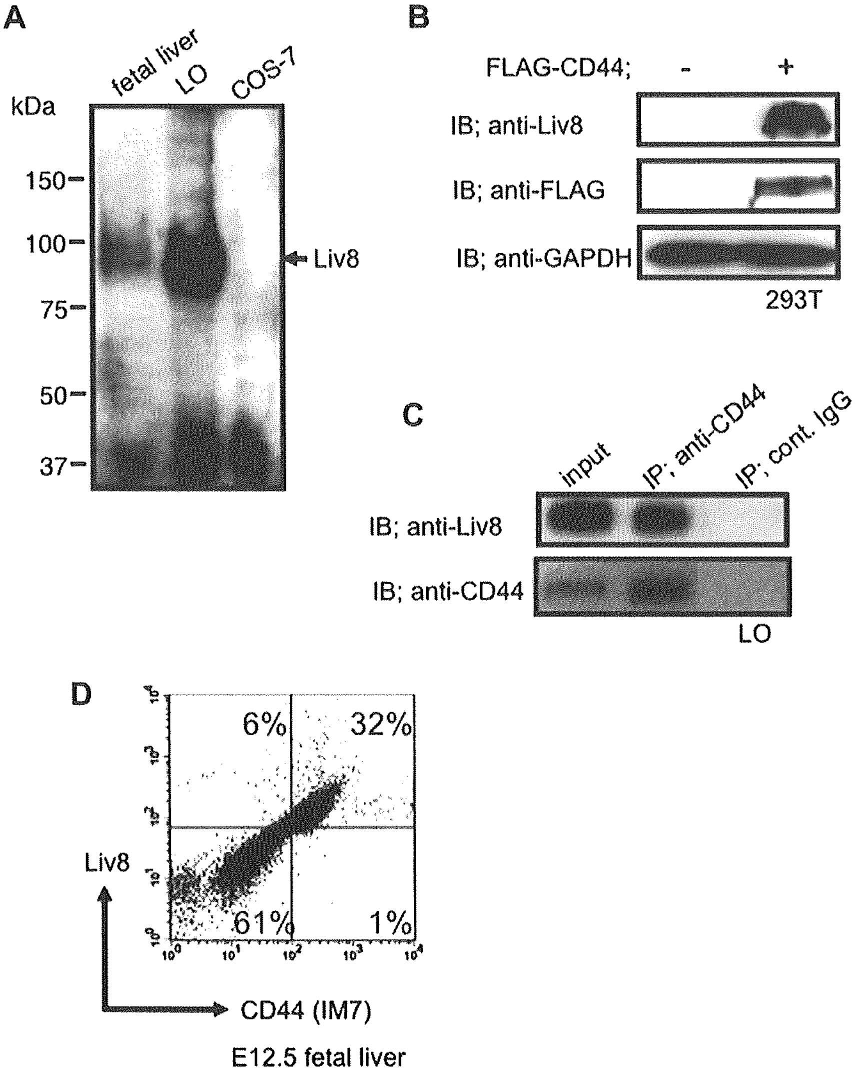

Fig. 2. Identification of Liv8 as CD44. (A) Western blot analysis of Liv8-antigen in fetal liver, LO, and COS-7 cells. (B) FLAG-CD44 or FLAG-peptide alone were expressed in 293T cells. Proteins were separated by SDS–PAGE, and immunoblot was performed with anti-Liv8, anti-CD44 (IM7), and anti-GAPDH antibodies. (C) LO membrane fraction was subjected to immunoprecipitation with anti-CD44 (KM114) antibody or control IgG conjugated resin. Proteins bound to the resin were separated by SDS–PAGE and immunoblotted with anti-Liv8 and anti-CD44 (IM7) antibodies. (D) E12.5 fetal liver cells were stained with FITC-conjugated anti-CD44 and biotin-conjugated anti-Liv8 antibody. The cells were further incubated with allophycicyanin-conjugated streptavidin, and analyzed by FACS. The panel illustrates a plot of fetal liver cells; axes indicate relative logarithmic fluorescence units for FITC (*x*-axis) and allophycicyanin (*y*-axis).

tification of Liv8$^+$ cells of each regions revealed that cells in R1 expressed Liv8/CD44 at high rate, and the rate was decreased gradually from R1 to R5. These results indicate that Liv8/CD44 antigen expression declines gradually during erythropoiesis.

Hematopoiesis-dependent expression of Liv8/CD44 in murine hepatoblasts

To examine the relationship between hematopoiesis and the expression of Liv8/CD44 in hepatoblasts, we utilized *AML1*$^{-/-}$ mice at E11.5, which lack definitive hematopoiesis [13,18]. Interestingly, Liv8/CD44 was expressed in wild-type fetal liver, however, it was completely lost in *AML1*$^{-/-}$ fetal liver (Fig. 4A). On the other hand, Liv8/CD44 was expressed in both wild-type and *AML1*$^{-/-}$ AGM regions (Fig. 4B). These results indicate that an inflow of Liv8/CD44-positive hematopoietic precursor cells into fetal liver is required for the induction of Liv8/CD44 expression in hepatoblasts at E11.5.

Discussion

In the present work, we identified Liv8 as an adhesion molecule CD44 (Fig. 2). We showed that Liv8/CD44 is expressed in hepatoblasts besides hematopoietic progenitor cells in fetal liver (Fig. 1), and the expression of Liv8/CD44 is co-localized with HA in fetal liver at E11.5 (Fig. 3). Furthermore, Liv8/CD44 expression in hepatoblasts is induced in a hematopoiesis-dependent manner (Fig. 4). Our results suggest that Liv8/CD44-positive hematopoietic cells from AGM region to fetal liver induce Liv8/CD44 expression in hepatoblasts at E11.5, and Liv8/CD44 link between hematopoietic cells and hepatoblasts through HA.

CD44 was identified as the major receptor for HA, a component of ECM [16]. It was reported that CD44 expressed by human CD34$^+$ hematopoietic cells and HA expressed in bone marrow sinusoid endothelium and the endosteum region, are essential for homing and repopulation [19]. In the previous and present study, we showed hematopoietic progenitor cells in fetal liver expressed

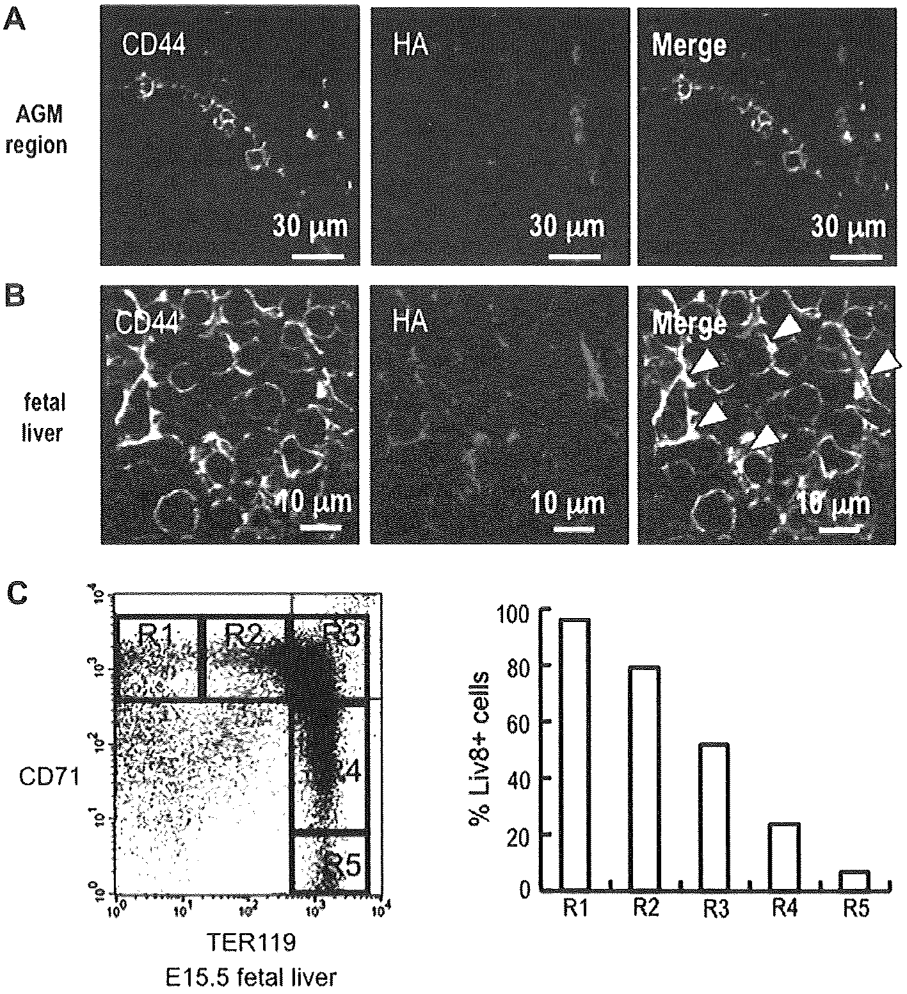

Fig. 3. Expression of Liv8/CD44 and HA in fetal livers. (A,B) Transverse frozen sections of AGM region and fetal liver at E11.5 were stained with anti-Liv8 antibody (green) and HABP (red). Arrowheads indicate co-localization of Liv8-antigen and HA. Scale bars indicate 30 μm (A) and 10 μm (B), respectively. (C) Mouse fetal liver cells were freshly isolated from E15.5 embryos and triple-stained with FITC-conjugated anti-CD71 antibody, PE-conjugated anti-TER119 antibody, and biotin-conjugated anti-Liv8 antibody. Binding of biotin-conjugated anti-Liv8 antibody was detected by using allophycicyanin-conjugated streptavidin. The left panel illustrates a plot of fetal liver cells; axes indicate relative logarithmic fluorescence units for PE (x-axis) and FITC (y-axis). Regions R1 to R5 are defined by characteristic staining pattern of cells (R1; CD71^{high}TER119^{low}, R2; CD71^{high}TER119^{meddle}, R3; CD71^{high}TER119^{high}, R4; CD71^{meddle}TER119^{high}, R5; CD71^{low}TER119^{high}). Each region was quantified for expression of Liv8-antigen (right panel).

Liv8/CD44 [11] and HA co-localized with Liv8/CD44 in fetal livers (Fig. 3). Therefore hematopoietic progenitor cells may flow into fetal liver and colonize there through the interaction between Liv8/CD44 and HA.

In this study, we found that the expression of Liv8/CD44 decreased gradually during erythropoiesis (Fig. 3). Downregulation of CD44 was shown in human bone marrow cells during erythroid development and implicated in promotion of cell migration [20]. Therefore downregulation of Liv8/CD44 may contribute for de-adhesion and departure of hematopoietic cells from its niche in fetal liver.

CD44 was reported to be a signal transmitter into the cells, and a growth factor presentation molecule [21]. CD44 can be cleaved within the transmembrane domain leading to release of the CD44 cytoplasmic tail domain into the cytoplasm. The resulting CD44 cytoplasmic tail fragment translocate to the nucleus, where it can function as a transcriptional activator. It may be interesting Liv8/CD44 transmit signals to each other to control their proliferation and differentiation through down stream molecules or by itself. Moreover, CD44 was reported to act as a linker that connects membrane-type 1 matrix metalloproteinase (MT1-MMP), which degrades ECM barriers during cancer invasion, to the actin cytoskeleton and to play a role in directing MT1-MMP to the migration front. Therefore Liv8-antigen/CD44 may also contribute for ECM reconstruction that is needed to enter and create hematopoietic niche in fetal liver.

Recently, Kon et al. showed that CD44 is a specific marker of small hepatocytes, which are hepatic progenitor cells in adult liver, and its expression is upregulated at the time small hepatocytes start to proliferate both *in vitro* and *in vivo* [22]. Moreover preliminary data of the authors suggest that HA is important for the proliferation of small hepatocytes, but keeps them in their less differentiated state. Therefore Liv8/CD44 may play important role in the proliferation and/or differentiation of hepatic progenitor cells in both embryo and adult.

It was reported that stimulation by cytokines such as OSM, transforming growth factor β, Tumor necrosis factor α, IL-6 and IL-8 induce the expression of CD44 in various cells [23–25]. In our preliminary experiment, the expression of Liv8/CD44 did not differ significantly between WT and OSM receptor knockout fetal liver at E11.5. Therefore expression of Liv8-antigen/CD44 may not be induced by OSM, or another candidate factor(s) may function in combination with OSM and have a redundancy in the induction of Liv8-antigen/CD44. It is expected that further investigation of the molecular mechanisms underlying the induction of Liv8/CD44 in hepatoblasts.

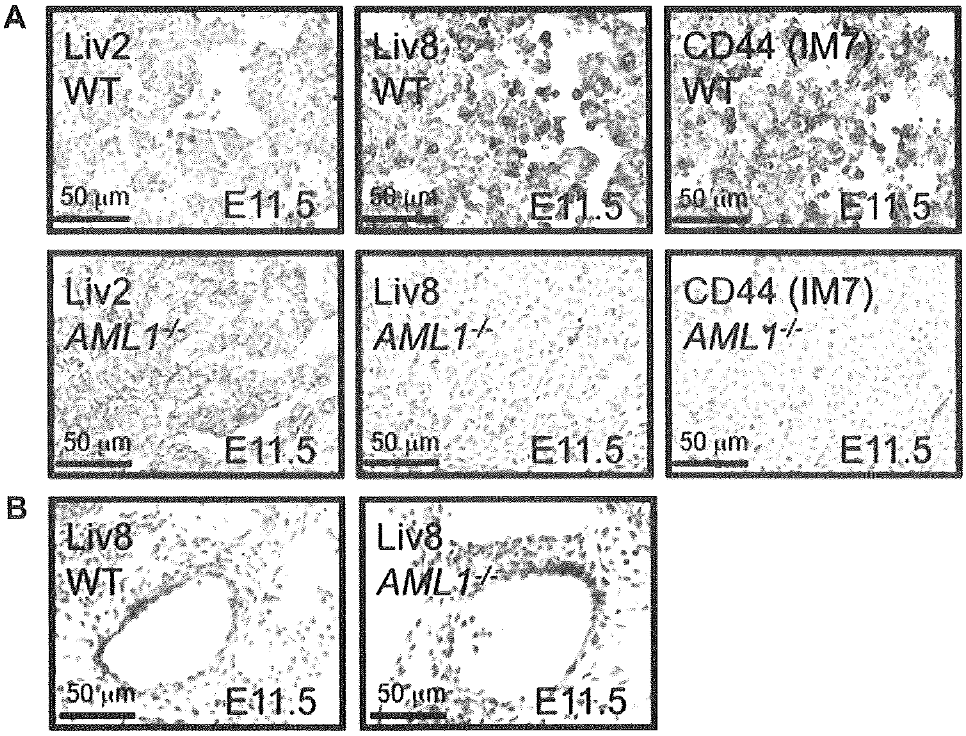

Fig. 4. Expression of Liv8/CD44 in WT and $AML^{-/-}$ fetal livers. (A,B) Transverse paraffin-sections of WT and $AML^{-/-}$ embryonic liver (A) and AGM region (B) at E11.5 were stained with anti-Liv2, anti-Liv8, and anti-CD44 (IM7) antibodies. Positive cells exhibit a brown precipitate. Nuclei were counterstained with hematoxylin (purple). Scale bars indicate 50 µm.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.149.

References

[1] G. Keller, G. Lacaud, S. Robertson, Development of the hematopoietic system in the mouse, Exp. Hematol. 27 (1999) 777–787.
[2] M. Hata, M. Nanno, H. Doi, S. Satomi, T. Sakata, R. Suzuki, T. Itoh, Establishment of a hepatocytic epithelial cell line from the murine fetal liver capable of promoting hemopoietic cell proliferation, J. Cell. Physiol. 154 (1993) 381–392.
[3] M. Nanno, M. Hata, H. Doi, S. Satomi, H. Yagi, T. Sakata, R. Suzuki, T. Itoh, Stimulation of in vitro hematopoiesis by a murine fetal hepatocyte clone through cell–cell contact, J. Cell. Physiol. 160 (1994) 445–454.
[4] A. Kamiya, T. Kinoshita, Y. Ito, T. Matsui, Y. Morikawa, E. Senba, K. Nakashima, T. Taga, K. Yoshida, T. Kishimoto, A. Miyajima, Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer, EMBO J. 18 (1999) 2127–2136.
[5] T. Kinoshita, T. Sekiguchi, M.J. Xu, Y. Ito, A. Kamiya, K. Tsuji, T. Nakahata, A. Miyajima, Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis, Proc. Natl. Acad. Sci. USA 96 (1999) 7265–7270.
[6] K.S. Zaret, Regulatory phases of early liver development: paradigms of organogenesis, Nat. Rev. Genet. 3 (2002) 499–512.
[7] A. Medvinsky, E. Dzierzak, Definitive hematopoiesis is autonomously initiated by the AGM region, Cell 86 (1996) 897–906.
[8] S. Hata, M. Namae, H. Nishina, Liver development and regeneration: from laboratory study to clinical therapy, Dev. Growth Differ. 49 (2007) 163–170.
[9] T. Watanabe, K. Nakagawa, S. Ohata, D. Kitagawa, G. Nishitai, J. Seo, S. Tanemura, N. Shimizu, H. Kishimoto, T. Wada, J. Aoki, H. Arai, T. Iwatsubo, M. Mochita, M. Satake, Y. Ito, T. Matsuyama, T.W. Mak, J.M. Penninger, H. Nishina, T. Katada, SEK1/MKK4-mediated SAPK/JNK signaling participates in embryonic hepatoblast proliferation via a pathway different from NF-kappa B-induced anti-apoptosis, Dev. Biol. 250 (2002) 332–347.
[10] T. Wada, N. Joza, H.M. Cheng, T. Sasaki, I. Kozieradzki, K. Bachmaier, T. Katada, M. Schreiber, E.F. Wagner, H. Nishina, J.M. Penninger, MKK7 couples stress signaling to G2/M cell cycle progression and cellular senescence, Nat. Cell Biol. 6 (2004) 215–226.
[11] I. Sakaida, S. Terai, N. Yamamoto, K. Aoyama, T. Ishikawa, H. Nishina, K. Okita, Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice, Hepatology 40 (2004) 1304–1311.
[12] N. Yamamoto, S. Terai, S. Ohata, T. Watanabe, K. Omori, K. Shinoda, K. Miyamoto, T. Katada, I. Sakaida, H. Nishina, K. Okita, A subpopulation of bone marrow cells depleted by a novel antibody, anti-Liv8, is useful for cell therapy to repair damaged liver, Biochem. Biophys. Res. Commun. 313 (2004) 1110–1118.
[13] H. Okada, T. Watanabe, M. Niki, H. Takano, N. Chiba, N. Yanai, K. Tani, H. Hibino, S. Asano, M.L. Mucenski, Y. Ito, T. Noda, M. Satake, AML1($^{-/-}$) embryos do not express certain hematopoiesis-related gene transcripts including those of the PU.1 gene, Oncogene 17 (1998) 2287–2293.
[14] T. Hara, Y. Nakano, M. Tanaka, K. Tamura, T. Sekiguchi, K. Minehata, N.G. Copeland, N.A. Jenkins, M. Okabe, H. Kogo, Y. Mukouyama, A. Miyajima, Identification of podocalyxin-like protein 1 as a novel cell surface marker for hemangioblasts in the murine aorta–gonad–mesonephros region, Immunity 11 (1999) 567–578.
[15] H. Kishimoto, K. Nakagawa, T. Watanabe, D. Kitagawa, H. Momose, J. Seo, G. Nishitai, N. Shimizu, S. Ohata, S. Tanemura, S. Asaka, T. Goto, H. Fukushi, H. Yoshida, A. Suzuki, T. Sasaki, T. Wada, J.M. Penninger, H. Nishina, T. Katada, Different properties of SEK1 and MKK7 in dual phosphorylation of stress-induced activated protein kinase SAPK/JNK in embryonic stem cells, J. Biol. Chem. 278 (2003) 16595–16601.
[16] A. Aruffo, I. Stamenkovic, M. Melnick, C.B. Underhill, B. Seed, CD44 is the principal cell surface receptor for hyaluronate, Cell 61 (1990) 1303–1313.
[17] J. Zhang, M. Socolovsky, A.W. Gross, H.F. Lodish, Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: functional analysis by a flow cytometry-based novel culture system, Blood 102 (2003) 3938–3946.
[18] T. Okuda, J. van Deursen, S.W. Hiebert, G. Grosveld, J.R. Downing, AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis, Cell 84 (1996) 321–330.
[19] A. Avigdor, P. Goichberg, S. Shivtiel, A. Dar, A. Peled, S. Samira, O. Kollet, R. Hershkoviz, R. Alon, I. Hardan, H. Ben-Hur, D. Naor, A. Nagler, T. Lapidot, CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow, Blood 103 (2004) 2981–2989.

[20] G.S. Kansas, M.J. Muirhead, M.O. Dailey, Expression of the CD11/CD18, leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans, Blood 76 (1990) 2483–2492.

[21] H. Ponta, L. Sherman, P.A. Herrlich, CD44: from adhesion molecules to signalling regulators, Nat. Rev. Mol. Cell Biol. 4 (2003) 33–45.

[22] J. Kon, H. Ooe, H. Oshima, Y. Kikkawa, T. Mitaka, Expression of CD44 in rat hepatic progenitor cells, J. Hepatol. 45 (2006) 90–98.

[23] M. Barshishat, A. Ariel, L. Cahalon, Y. Chowers, O. Lider, B. Schwartz, TNFalpha and IL-8 regulate the expression and function of CD44 variant proteins in human colon carcinoma cells, Clin. Exp. Metastasis 19 (2002) 327–337.

[24] J. Cichy, E. Pure, Oncostatin M and transforming growth factor-beta 1 induce post-translational modification and hyaluronan binding to CD44 in lung-derived epithelial tumor cells, J. Biol. Chem. 275 (2000) 18061–18069.

[25] T. Vincent, N. Mechti, IL-6 regulates CD44 cell surface expression on human myeloma cells, Leukemia 18 (2004) 967–975.

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Review Article

Liver development: lessons from knockout mice and mutant fish

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The liver is an organ with vital functions, including the processing and storage of nutrients, maintenance of serum composition, detoxification and bile production. Over the last 10 years, there have been major advances in our understanding of the molecular and cellular mechanisms underlying liver development. These advances have been achieved through the use of knockout mice as well as through forward-genetics studies employing mutant fish. The examination of many such murine and piscine mutants with defects in liver formation and/or function have pinpointed numerous factors crucial for hepatic cell differentiation and growth. In addition, these studies have permitted the identification of several important liver-specific markers that allow the contributions of variouscell types to hepatogenesis to be monitored. This review summarizes our current state of knowledge of the shared molecular mechanisms that underlie liver development in species as diverse as fish and mice. A better molecular understanding of liver formation may provide new insights into both normal liver biology and liver disease.

Key words: antibody, knockout mice, liver development, medaka, zebrafish

INTRODUCTION

THE LIVER PLAYS a central role in metabolic homeostasis because this organ is responsible for the metabolism, synthesis, storage and redistribution of nutrients such as carbohydrates, fats and vitamins. The liver is also the main detoxifying organ of the body, removing waste and xenobiotics through metabolic conversion and biliary excretion. Most of these functions are carried out by hepatocytes and bile duct cells. The common progenitor of these cell types, the hepatoblast, arises during early embryogenesis at a specific location in the embryonic endoderm. Hepatoblasts go on to either self-renew or proliferate and differentiate into hepatocytes and bile duct cells under the influence of a wide spectrum of genes.[1-3] Owing to the essential nature of liver functions, mutation of many of these genes results in embryonic lethality, which makes it difficult to use genetic screening to isolate developmental mutants with liver defects. Instead, reverse-genetics studies using mice and forward-genetics approaches using small aquarium fish such as zebrafish and medaka have been successfully employed to reveal much information about regulatory genes that are crucial for liver development (Table 1). Another barrier to analyzing mechanisms of liver development has been a dearth of liver-specific markers. However, in recent years, several molecules that are expressed specifically in fetal liver cells have been identified. In this review, we discuss several important molecular aspects of liver development as revealed by the use of these markers and mutants.

STAGES OF LIVER DEVELOPMENT

EMBRYONIC LIVER DEVELOPMENT occurs in multiple stages that are governed by hormonal factors as well as by intercellular and matrix-cellular interactions. In mice, liver ontogeny initiates around embryonic day 9 (E9), when epithelial cells of the foregut endoderm interact with the cardiogenic mesoderm and commit to becoming the liver primordium[4] (Fig. 1). The liver primordium proliferates and invades the mesenchyme of the septum transversum to give rise to the hepatic codes

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Table 1 Genes and mutations affecting liver formation and function

Mouse

Genes	Description	Knockout mouse liver phenotype
Bmp4	TGF-β family	Delayed growth of the hepatic bud, defect in hepatoblast proliferation
Hhex	Transcriptional repressor	Defect in hepatoblast differentiation, lack of Hnf4α Hnf6 expression
RelA	NF-κB subunit	Massive liver degeneration and apoptosis
Ikkβ	NF-κB signaling molecule	Massive liver degeneration and apoptosis
NEMO	NF-κB signaling molecule	Massive liver degeneration and apoptosis
Tbk1	NF-κB signaling molecule	Massive liver degeneration and apoptosis
Pik3r1	PI3-kinase subunits	Hepatocyte necrosis
Mkk4	MAPKK	Impaired hepatoblast proliferation
Mkk7	MAPKK	Impaired hepatoblast proliferation
c-jun	AP-1 transcription factor	Defect in liver development
Tbx3	T-box transcription factor	Impaired hepatoblast proliferation
Hlx	Homeobox transcription factor	Impaired hepatic development
Xbp1	X-box transcription factor	Hypoplastic fetal liver
C/EBPα	Transcription factor	Failure of normal liver development
K-ras	GTPase	Defect in the fetal liver microenvironment
Hnf6	Transcription factor	Absence of hepatic artery branches

Zebrafish

Mutation	Description	Liver phenotype
prometheus (Wnt2bb)	Wnt2bb	Defects in liver specification

Medaka

Genes		Liver phenotype
hirame	Not identified	Defect in hypoblast convergence
fukuwarai	Not identified	Absence of the hepatic bud
sakura	Not identified	Absence of the hepatic bud
mochizuki	Not identified	Lack of foxA3 expression
akatsuki	Not identified	Lack of foxA3 expression
akebono	Not identified	Lack of foxA3 expression
kakurenbo	Not identified	Small and mislocated liver
hiohgi	Not identified	Small liver
kamifusen	Not identified	Malformed liver
origami	Not identified	Malformed liver
kendama	Not identified	Inverted positions of liver and gall bladder
dendendaiko	Not identified	Inverted positions of liver and gall bladder
hanetsuki	Not identified	Inverted positions of liver and gall bladder
akane	Not identified	Deep red bile
suou	Not identified	Light red bile
ominaeshi	Not identified	Colorless bile
ukon	Not identified	Failure to metabolize PED6 (phospholipase A2 substrate)
aonibi	Not identified	Failure to metabolize PED6 (phospholipase A2 substrate)
uguisucha	Not identified	Failure to metabolize PED6 (phospholipase A2 substrate)

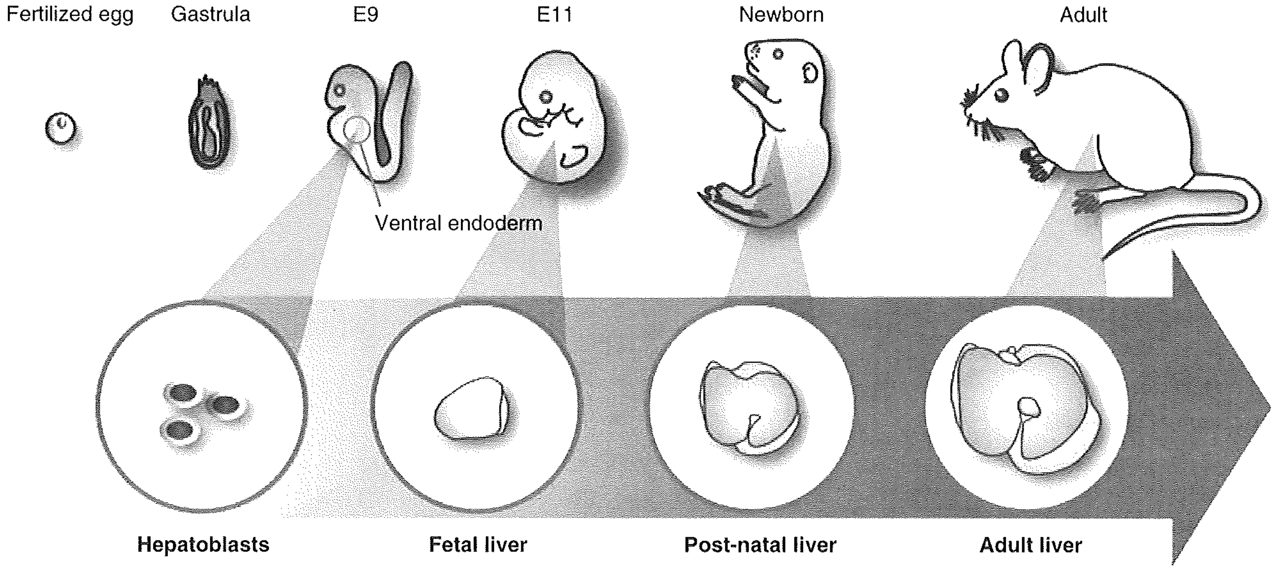

Figure 1 Overview of the stages of murine liver development. The fertilized egg develops into embryo through gastrulation and organogenesis begins. By embryonic day 9 (E9), the ventral endoderm generates hepatoblasts. By E11, the fetal liver forms and becomes the major site of hematopoiesis. After birth, the post-natal liver functions mainly in metabolism, a capacity maintained in the adult.

and hepatic bud at E9.5. At E10.5–12.5, the fetal liver takes over hematopoiesis from the yolk sac and aorta-gonad-mesonephros (AGM) region. This switch in hematopoietic organ represents a critical checkpoint in murine embryogenesis. The next major stage of liver development occurs around E14.5 when bipotential hepatoblasts present in the hepatic bud differentiate into hepatocytes and bile duct cells.[5,6] These cells continue to proliferate and eventually cooperate to form the post-natal liver. The liver's main function then switches from hematopoiesis to metabolism, a function that also dominates in the adult liver.

KNOCKOUT MOUSE MODELS OF LIVER DEVELOPMENT

A VARIETY OF knockout mouse models have been used to elucidate different aspects of liver development, including: (i) the development of hepatoblasts from multipotential endodermal stem cells; (ii) the survival and proliferation of hepatoblasts; and (iii) the differentiation of bile duct cells and hepatocytes from hepatoblasts (Fig. 2). Fibroblast growth factors (FGF) and bone morphogenic proteins (BMP) are essential for hepatic bud formation and for the development of hepatoblasts from endodermal stem cells. For example, mice deficient for BMP-4 exhibit delayed growth of the hepatic bud.[7] Homeobox genes are also critical, as the endoderm-specific homeobox gene, *Hhex*, has been found to be essential for proper hepatoblast differentiation. *Hhex* is expressed in the endoderm before liver induction and is necessary for the expression of other genes involved in liver development.[8–10] In *Hhex* knockout mice, expression of the *Hnf4α* and *Hnf6* genes, which are required for the differentiation of bile duct cells and hepatocytes from hepatoblasts, is lost.[11]

Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine that elicits a wide range of biological responses, including tumor necrosis, inflammation, cellular differentiation, proliferation and apoptosis (Fig. 3). In the fetal liver, TNF-α engagement of TNF receptor 1 (TNFR1) activates three separate signaling pathways: (i) activation of a cell death signal; (ii) activation of NF-κB signaling that opposes the cell death signal and guarantees cell survival; and (iii) activation of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) signaling that induces cell proliferation. Knockout mice lacking genes involved in NF-κB signaling are embryonic lethal and exhibit massive liver degeneration and apoptosis during midgestation at E12.5–16. Examples include mice deficient for the RelA subunit of transcription factor nuclear factor-κB (NF-κB) (die at E15–16),[12] IKKβ (die at E12.5–14),[13–15] NEMO (die at E12.5–13.0),[16] or TANK-

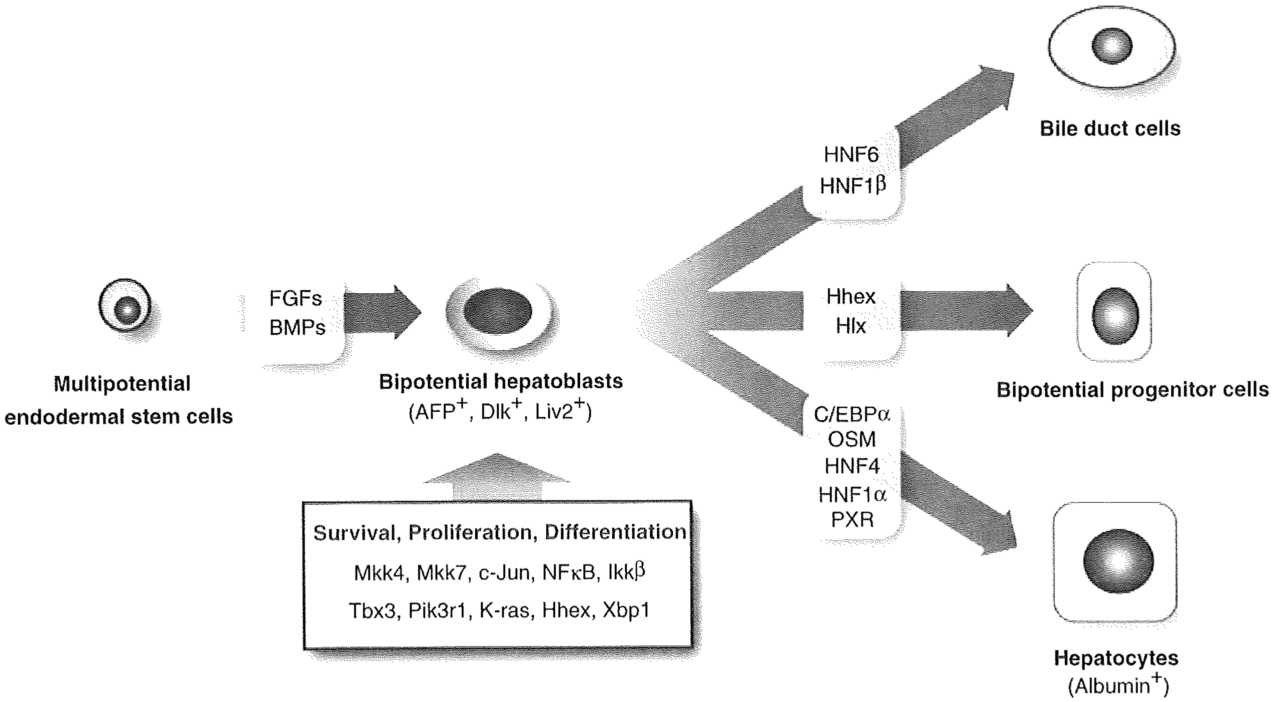

Figure 2 Factors influencing the development, survival, proliferation and differentiation of murine hepatic cells. Under the influence of fibroblast growth factors (FGF) and bone morphogenic proteins (BMP), multipotential endodermal stem cells generate bipotential hepatoblasts. Under the influence of the indicated genes, the hepatoblasts either self-renew or differentiate into bile duct cells and hepatocytes. Many of the indicated factors are discussed in the text. See Table 1 for information on other markers. AFP, α-fetoprotein.

binding kinase (TBK1; die at E12.5–14.5).[17] Importantly, the embryonic lethality and liver cell death observed in RelA-, IKKβ- or TBK1-deficient embryos can be rescued by the simultaneous inactivation of TNFR1. These results suggest that developing liver cells are routinely exposed to TNF-α produced by hematopoietic cells, and that the death signaling mediated by TNF-α must be countered by NF-κB signaling to ensure continued liver development. Another gene required for hepatocyte survival is *Pik3r1*, which encodes regulatory subunits of phosphoinositide-3-kinase (PI3K). Knockout mice lacking Pik3r1 expression show massive hepatocyte death and perinatal lethality.[18]

c-Jun N-terminal kinase is primarily activated in response to a variety of cellular stresses, including metabolic poisons, DNA damage, changes in osmolarity, heat shock, and the presence of the inflammatory cytokines interleukin (IL)-1 and TNF-α. Activated JNK phosphorylates c-Jun and thereby promotes the formation of the c-Jun/Fos heterodimer that constitutes the AP-1 transcriptional complex, which is a key regulator of gene expression. JNK is also directly activated by MKK4 (also called SEK1) and MKK7, which are activated in response to the binding of hepatocyte growth factor (HGF) to its receptor c-Met. We and several other groups have used gene-targeting to disrupt the *Mkk4* gene in mice and have found that MKK4-deficient embryos display severe anemia and die at E10.5–12.5.[19-23] Although vasculogenesis and hematopoiesis from yolk sac precursors are normal in MKK4-deficient embryos, liver formation is severely impaired. Hepatocyte numbers are greatly reduced in MKK4-deficient embryos due to massive hepatoblast apoptosis at E12.5. Embryos with a disrupted *c-jun* gene also display defective liver organization and die at E11.5–15.5.[24,25] MKK7-deficient mice show impaired liver formation at E11.5–12.5 as well as reduced c-Jun phosphorylation and decreased expression of the G2/M cell-cycle kinase Cdc2.[26] This latter result indicates that JNK signaling leading to c-Jun phosphorylation and thus Cdc2 regulation is vital for hepatoblast proliferation in the developing liver.

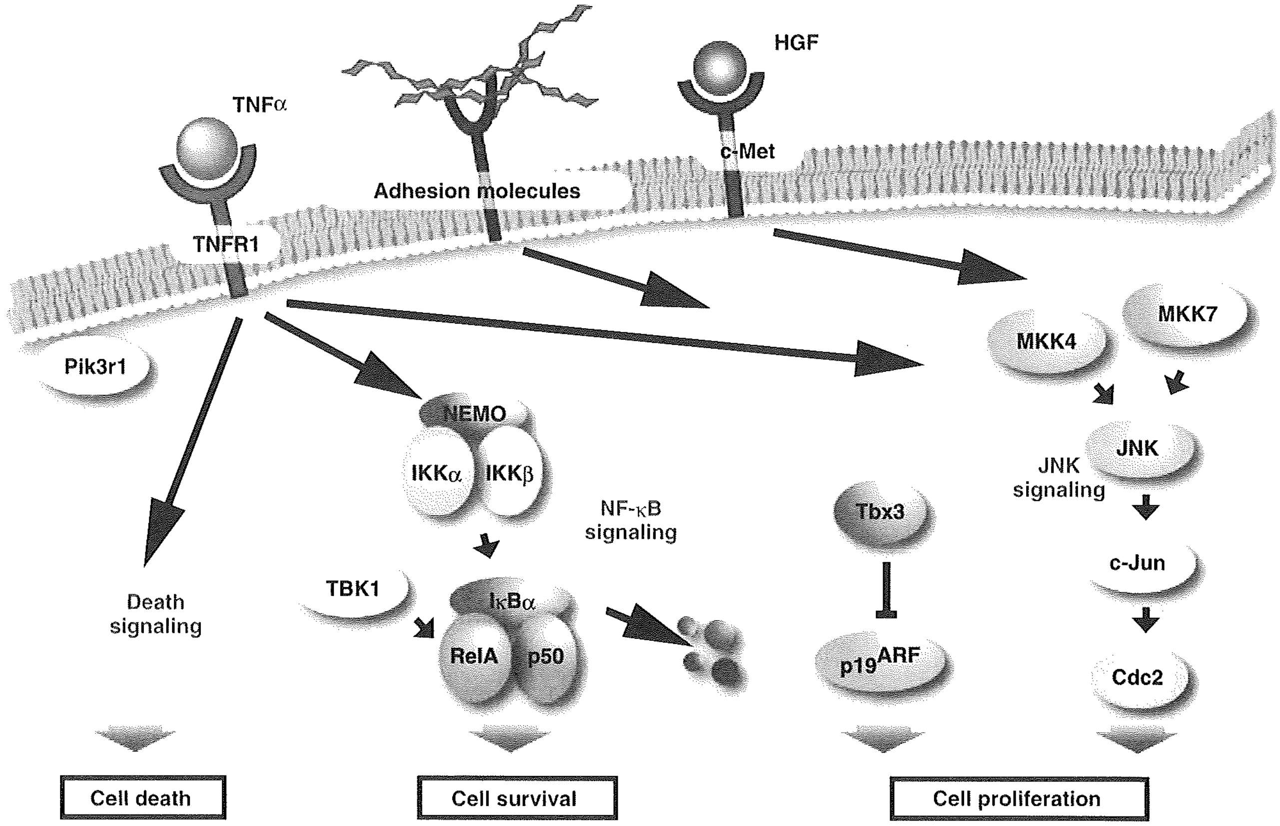

Figure 3 A proposed model for signaling pathways in murine hepatoblasts. Engagement of tumor necrosis factor-α engagement of TNF receptor 1 (TNFR1) by tumor necrosis factor-α elicits a wide range of biological responses, including cell death, survival and proliferation. In hepatoblasts, the induction of cell-death signaling, nuclear factor-κB (NF-κB) activation and c-Jun N-terminal kinase (JNK) activation are simultaneously mediated through TNFR1. JNK is also activated by the binding of hepatocyte growth factor (HGF) to its receptor c-Met. NF-κB activation requiring all the signaling elements shown here protects hepatoblasts against cell death, whereas JNK activation leading to c-Jun phosphorylation and *cdc2* gene expression drives cell proliferation. The Tbx3 transcription factor suppresses $p19^{ARF}$ expression, allowing hepatoblast proliferation and, subsequently, differentiation.

Another transcription factor important for liver development is the T-box gene, *Tbx3*. *Tbx3* is the first T-box gene implicated in yolk sac development, and mutations in this gene cause Ulnar-Mammary syndrome. Knockout mice lacking *Tbx3* exhibit embryonic lethality at E12.5 due to impaired yolk sac formation.[27] *Tbx3* also controls the fate of hepatoblasts during liver development, because Tbx3-deficient hepatoblasts show severe defects in proliferation and are abnormally biased towards bile duct cell differentiation.[28] At the molecular level, deletion of *Tbx3* results in increased expression of the tumor suppressor $p19^{ARF}$, which in turn induces growth arrest in hepatoblasts and activates the bile duct cell differentiation program. Thus, *Tbx3* plays an important role in controlling the proliferation and cell-fate determination of hepatoblasts by suppressing $p19^{ARF}$ expression.

MUTANT FISH MODELS OF LIVER DEVELOPMENT

OVER THE LAST decade, studies in rats and mice have greatly expanded the list of molecules known to contribute to liver development. However, identification of novel factors using mammalian model organisms is inefficient and expensive compared with studies of other vertebrate model organisms such as zebrafish (*Danio rerio*) and medaka (*Oryzia latipes*). These small fish can be bred in large numbers and require little cost and effort to maintain. High-throughput screens based on these models are already yielding a wealth of new information on liver gene functions.[29] In zebrafish, random chemical mutagenesis mediated by *N*-acethyl *N*-nitrosourea (ENU) treatment is widely used in forward-genetics screening (Fig. 4). Another approach is to carry out ENU-mediated mutagenesis of a transgenic

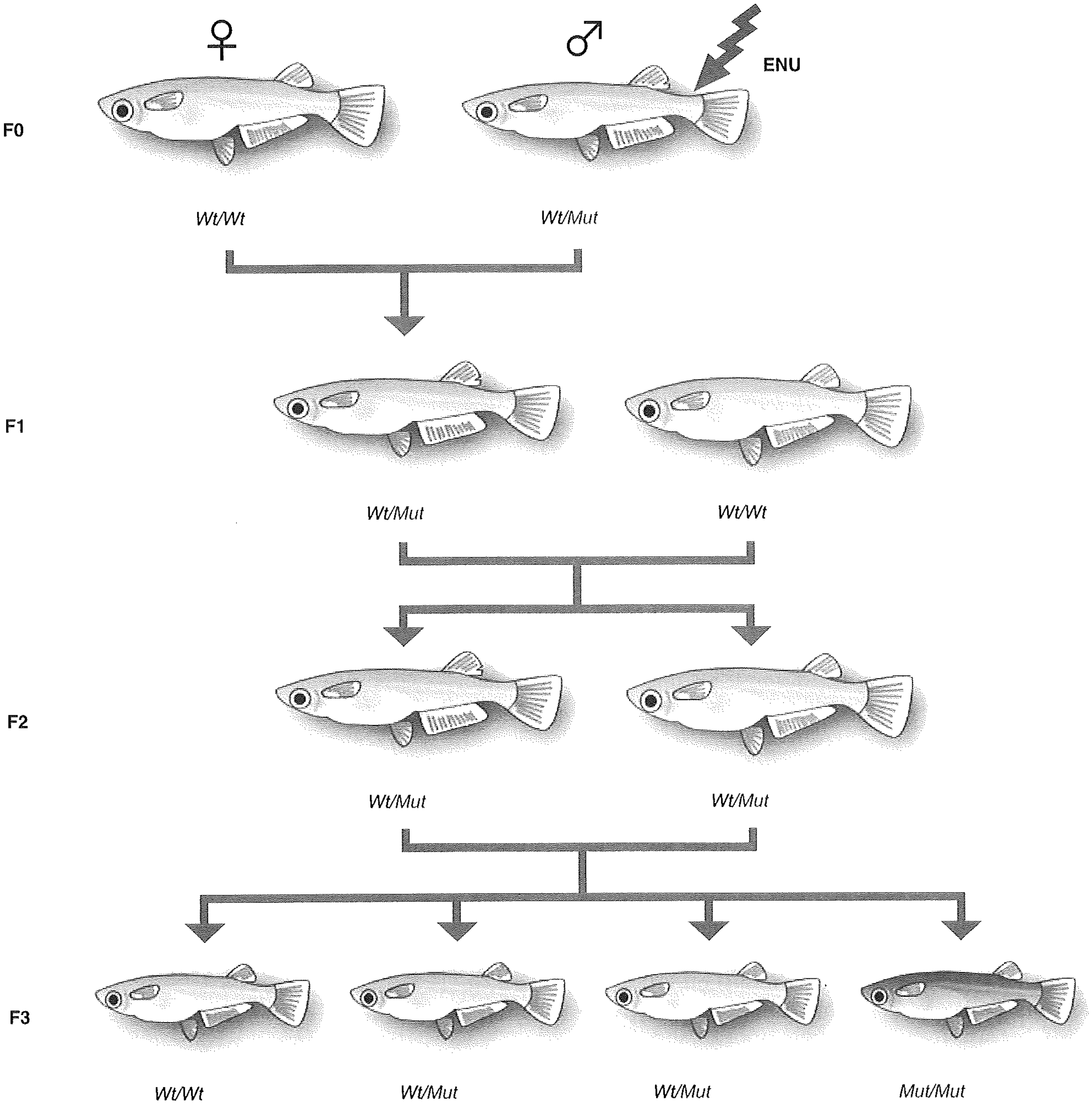

Figure 4 Scheme illustrating the mutagenesis and breeding of small fish to identify recessive mutations. Male fish are treated with *N*-acethyl *N*-nitrosourea (ENU) to induce point mutations (*Mut*) in the germline. The treated males are bred to untreated, wild-type females (*Wt*) to fix the mutations in the resulting progeny. The F_1 progeny are bred with Wt fish and the resulting F_2 progeny are intercrossed. The F_3 clutches contain 25% phenotypically abnormal offspring.

zebrafish line that expresses green fluorescent protein (GFP) in the developing endoderm (Fig. 5).[30] This latter method was used by Ober *et al.* to identify zebrafish with mutations in the *Prometheus* (*prt*) gene. Some *prt* mutants show a failure to develop hepatic tissue at 28 h post-fertilization, whereas other *prt* mutants are able to do so at later stages.[31] Positional cloning revealed that the *prt* gene encodes the important developmental

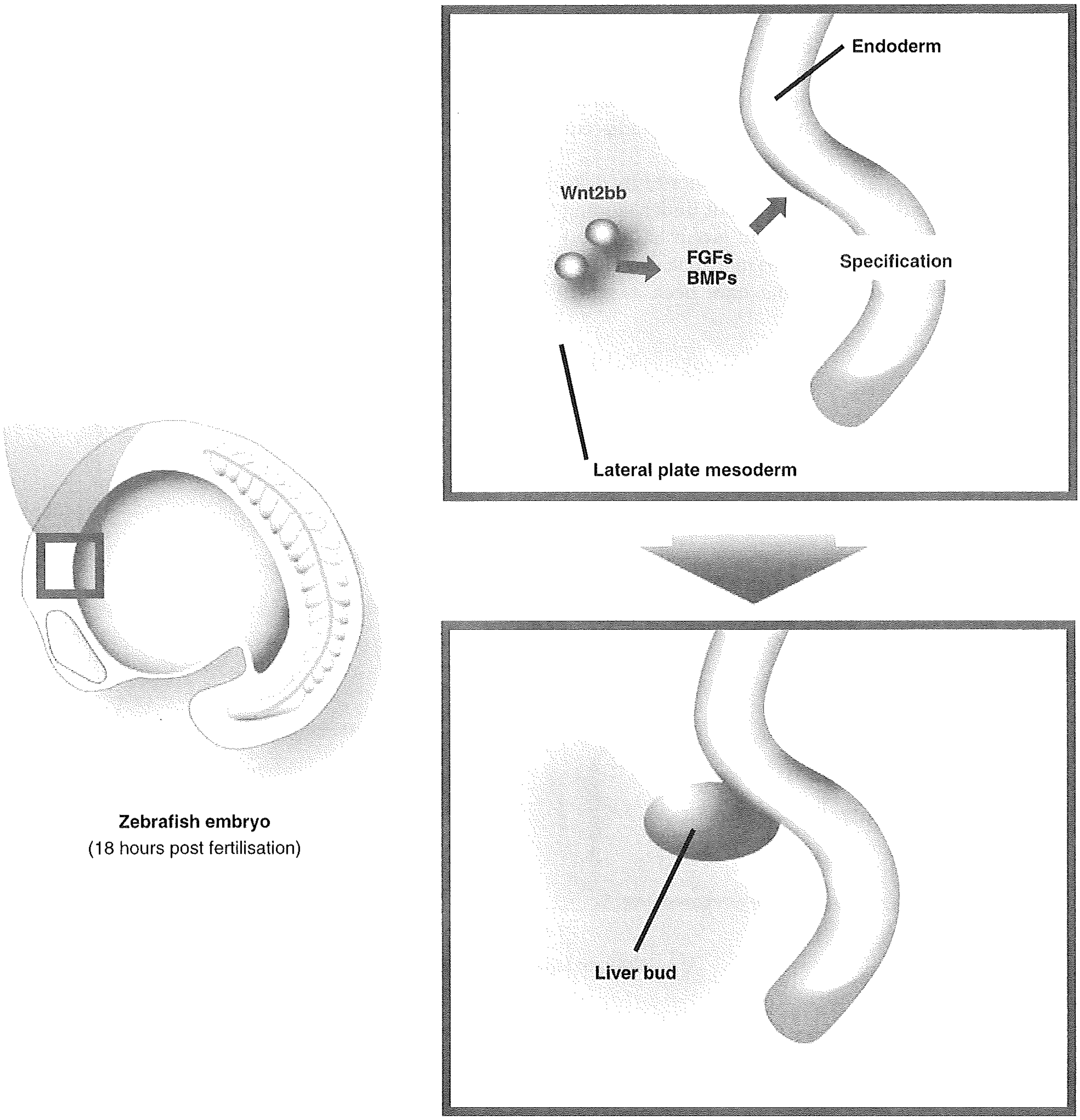

Figure 5 ENU-EGFP mutant fish screening method. A transgenic zebrafish line expressing EGFP in developing endoderm is treated with ENU to induce random mutations. The mutagenized fish are then induced to undergo hepatogenesis. This method was used to establish the role of the Wnt signaling pathway in zebrafish liver development. The lateral plate mesoderm that associates with the liver bud-forming region of endodermal epithelium emits a Wnt2bb signal. BMP, bone morphogenic proteins; FGF, fibroblast growth factors.