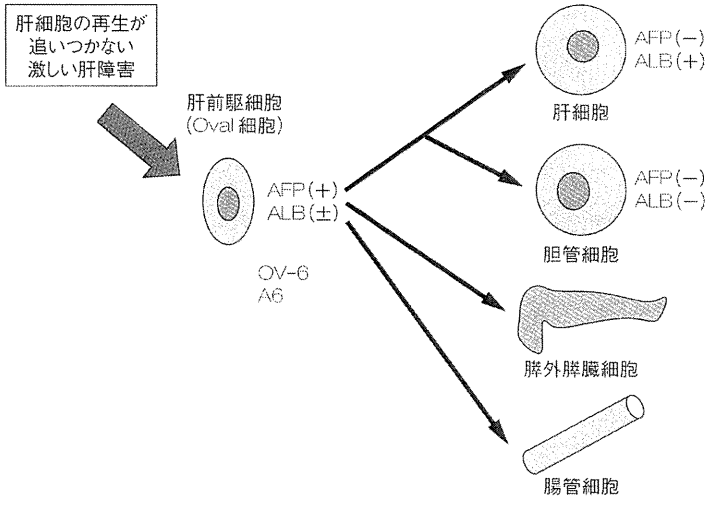
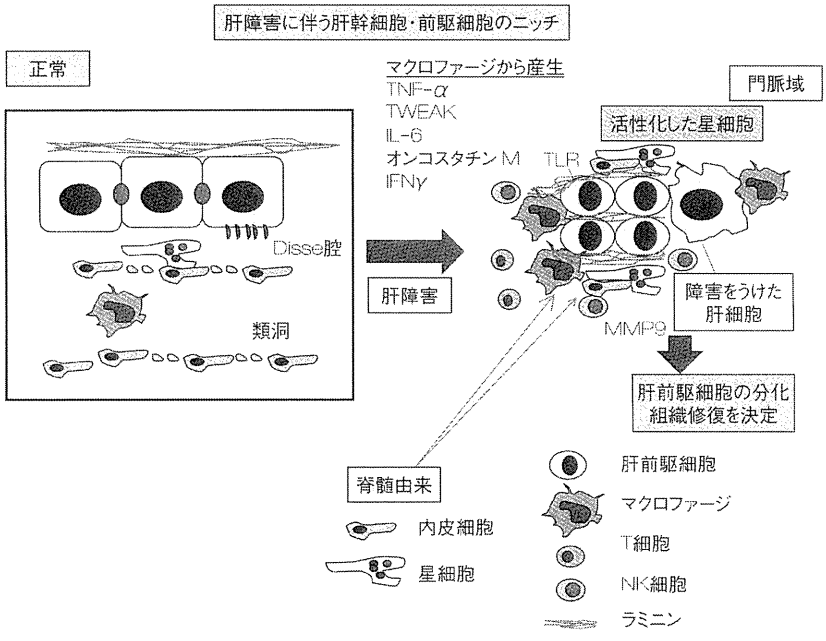
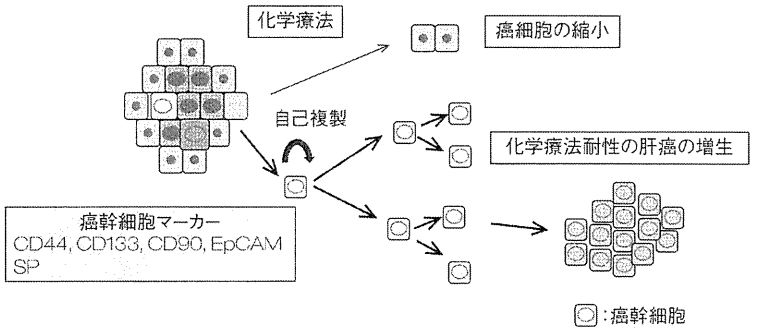


図❷ 肝前駆細胞（Oval 細胞）の分化〔AAF（Acetylaminofluorene）/PH（partial hepatectomy：部分肝切除）モデルより〕
AAF を投与し肝再生ができない．ALB：アルブミン



図❸ 肝前駆細胞の制御と分化ニッチ，マクロファージ
TLR：Toll-like receptor. MMP：matrix metalloproteinase

じめ多くの癌で見出されている．また，癌幹細胞の多くは薬物輸送蛋白を強発現しており，ヘキストを細胞外に排出することから，フローサイトメトリーによりほかの細胞と区別される[6]．また，幹細胞のもつ強い薬物排出能は，抗癌剤に対して耐性を示し，癌の再発の原因となる．したがって，癌の根治には癌幹細胞を除去することが重要である．癌幹細胞であるが，幹細胞としての性質をもつ細胞群であり，さまざまな分化能を示す．癌幹細胞は，化学療法に耐性の細胞群であり，その制御が癌治療に対する患者の予後を決める．癌幹細胞は正常の幹細



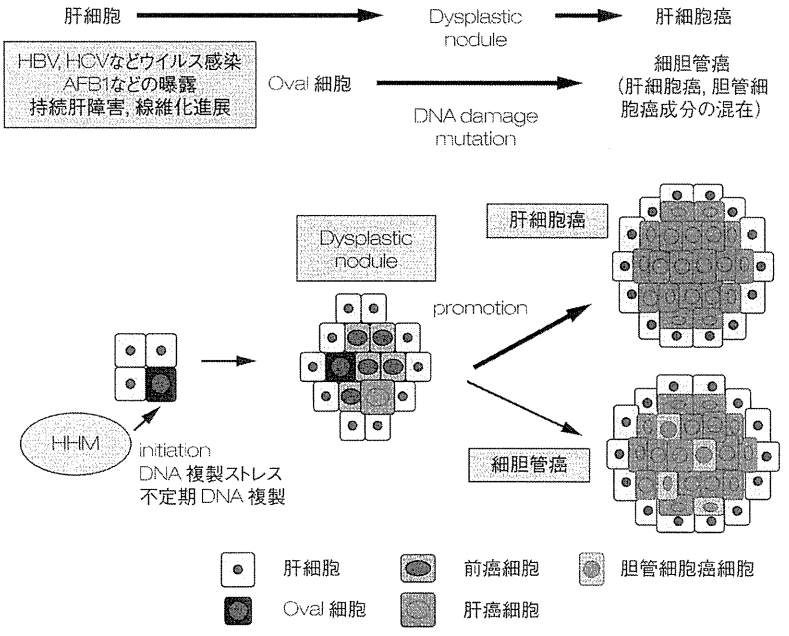
図❹　抗癌剤耐性から考えた癌幹細胞

胞と同じように“自己複製能と分化能”をもち，癌幹細胞は前駆細胞としてさまざまな癌細胞をつくり出す力があり，この癌幹細胞を制御することは癌の予後を決めるうえで重要である(図❹)．肝癌幹細胞マーカーとしては，CD133[7,8]，CD90 (Thy-1)[9]，epithelial cell adhesion molecule (EpCAM)[10]，CD13[11]などが肝癌幹細胞マーカーと報告されている．これらのマーカー陽性細胞を1×10^2～1×10^3個の細胞を使うことで，tumor initiating cellとしての機能を発揮した．これらの細胞の癌細胞株に含まれる頻度は，抗原の種類から0.1～90%とさまざまであり，完全に癌幹細胞を1つの抗体で排除することは困難なことが予測される[12]．

肝発癌

基本的な発癌機構として，前癌性病変のdysplastic noduleができ，さらに肝細胞癌が発生すると考えられる[13]．発癌は，アフラトキシンB1 (AFB1)，B型肝炎ウイルス (hepatitis B virus：HBV)，C型肝炎ウイルス (hepatitis C virus：HCV)，最近では脂質，糖代謝の異常による持続肝障害が癌化を誘導する(図❶，図❺)．その分子メカニズムは日々新たな知見が明らかになってきている．β-カテニンシグナルの亢進，p53の変異による失活，retinoblastoma (RB)の失活，mitogen-activated protein kinase (MAPK)シグナルの亢進，ストレス応答シグナルのheat shock protein (HSP) 27のリン酸化の変化，上皮増殖因子(epidermal growth factor：EGF)およびtransforming growth factor (TGF)-β経路の変化が知られている[14]．慢性炎症との関連では，nuclear factor (NF)-κB，インターロイキン (IL)-6，JAK/STATが重要である[15](図❸)．その他，phosphatase and tensin homolog (PTEN)およびmothers against decapentaplegic homolog (Smad)の肝臓特異的ノックアウト(KO)は胆管細胞癌を誘導することが知られている[16]．またβ-カテニンは，前駆細胞および肝癌の制御にかかわることなどが明らかになっている[17]．ウイルス発癌については，HBV，HCVについてであるが，HBVはDNAウイルスで宿主のDNAにintegrateされるが，HCVはされない．しかしながら両方のウイルスとも，肝細胞の細胞内シグナル伝達の異常を誘導することで，細胞の異常増殖を誘導させる[18]．Oval細胞は肝細胞と胆管上皮細胞への分化能をもつことから，この細胞に由来する肝癌は肝細胞，胆管細胞の両方の成分が混在する細胆管癌として注目をあびている[19～21]．

われわれは，肝幹細胞の分化制御機構の解明，肝臓の発生分化を制御する肝臓特異的なHelix-Loop-Helix (HLH)型転写制御因子(マスター遺伝子)の同定をめざし，研究をおこなってきた．肝胆膵領域におけるHLH型転写制御分子としては，hairy and enhancer of split (Hes)がよく知られている．さらに，Hesを制御する因子としてJagged1やNotch2が同定されているが，これらは胆管細胞消失症候群・アラジール (alagille)症候群の原因遺伝子である．E12蛋白をおとり蛋白としたtwo hybridスクリーニングにより，inhibitor of DNA binding (Id)とは異なる新規のdominant inhibitory型HLH型転写制御分子であるhuman homologue of maid



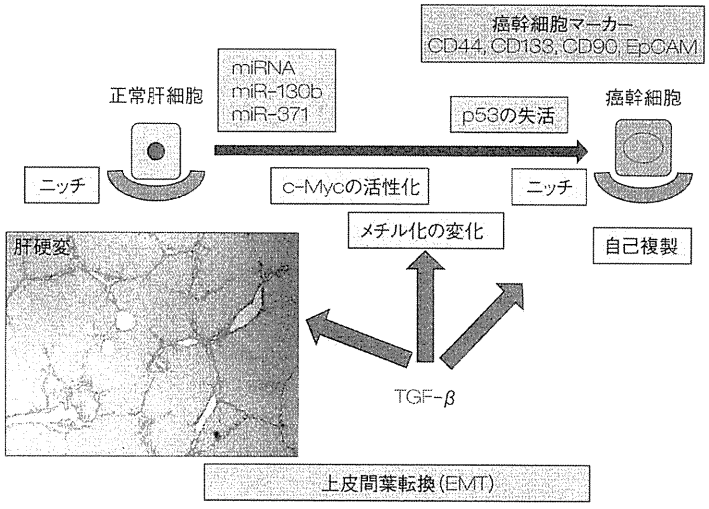
図❺ 肝発癌（肝細胞癌の発生，細胆管癌の発生）

(HHM) をクローニングした．HHM は 360 個のアミノ酸から形成される Id2 とくらべ大きい，新規の dominant inhibitory 型の HLH 型転写制御分子である．Maid は，生誕直後の 2 細胞期に発現が増加することがわかっていたが，さらにわれわれの解析から，HHM が肝細胞の分化成熟に関与する hepatocyte nuclear factor 4 (HNF4) の発現を特異的に制御し，2-アセチルアミノフルオレン (2-acetylaminofluorene：AAF) 肝部分切除モデルにおいて Oval 細胞の肝細胞の分化過程に出現する α-フェトプロテイン (AFP) 陽性の Foci に一致して HHM の発現が特異的に高いことが明らかとなった．また HHM は，肝細胞の分化を制御する転写因子 HNF4 を制御することも明らかになった[22]．HHM の肝発癌における解析をおこない，肝細胞癌における解析として，ラットコリン欠乏食モデルの解析において HHM の発現は placental glutathione S-transferase (GST-P) 陽性 Foci と一致し増加し，さらに HHM はヒトの肝前癌性病変や肝細胞癌の癌部で高発現することが明らかになった[23]．この肝発癌における機構として，HHM の強制発現により細胞の複製を促進し，肝前癌性病変 dysplatic nodule が発生に関与する可能性を考えている．また HHM は細胆管癌でも HHM 陽性の細胞の存在を確認しており，HHM は肝臓における幹細胞，癌幹細胞の発生を制御している可能性が示唆される．

一方で，CD44，CD133，CD90 (Thy-1)，aldehyde dehydrogenase (ALDH) などのマーカーについて，実際の 25 個の肝癌，4 個の肝芽腫，8 個の前癌性病変，19 個の肝炎組織について検討した．その結果，前癌性病変である dysplastic nodule において CD133 陽性かつ ALDH 陽性であったが，悪性所見がない慢性肝炎の組織でも CD133，CD44 は陽性であり，必ずしも肝細胞癌の特異マーカーとはいえない細胞であった[24]．これらの結果から，現状でみつかった癌幹細胞マーカーは，病理的な組織解析には使いにくい抗体であるかもしれない．

癌幹細胞の発生機構（初期化）また自己複製～未分化細胞な幹/前駆細胞が癌の起源か？　あるいは分化した細胞から癌が発生するか？～

癌幹細胞の起源については，まだ一定の見解が一致していない．癌幹細胞は，正常組織内の幹細胞や前駆細胞



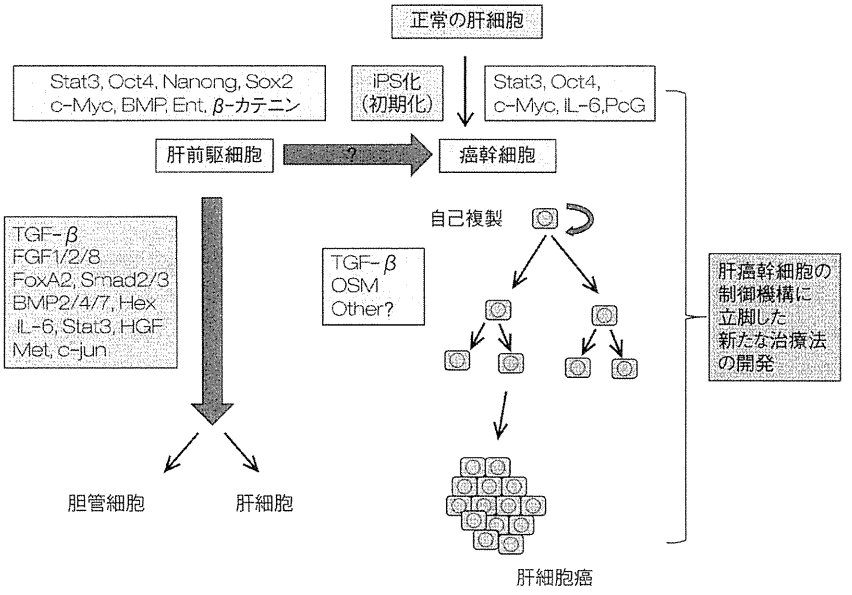
図❻　癌幹細胞の発生

から直接形質転換する[25]．あるいは上皮間葉転換（epithelial-mesenchymal transition：EMT）[26]の変化で幹細胞様に変化することが考えられる[27]．肝硬変症はEMTを起こしやすい状態であり，発癌を起こしやすい状態である[28]．肝硬変状態においては，EMTを制御するTGF-βシグナルが肝硬変で強く発現している[29]．

一方，分化した肝細胞にc-Mycを含むわずか4つの遺伝子を発現させることで多分化能をもったiPS細胞が生じることが示されており[30]，分化した細胞のリプログラミングが癌の発生においても重要であると考えられる．c-Mycで誘導されるmiR-371[31]やmiR-130bなどは癌幹細胞のマーカーのCD133の発現に非常に相関がある[32]．miR-130bなど癌幹細胞のマーカーのCD133の発現に非常に相関があるmiRNAもみつかってきている．一方で，EMTを制御するTGF-βシグナルがCD133などの発現などを誘導する知見も出てきている[8]．自己複製機構については依然として不明な部分が多いが，Polycombシグナルも重要であり[33]，最近，glycogen synthase kinase β（GSKβ）/β-カテニンを介した機構なども注目されている[34]（**図❻**）．

癌幹細胞ニッチ（niche）と治療への応用の可能性

肝癌幹細胞についての新たな知見が日々亢進されているが，肝細胞癌患者の根治術を考えるうえにおいて，肝癌幹細胞に注目した治療法を開発することは必須である．そのアプローチとしては，①癌幹細胞の自己複製を阻害する，②癌幹細胞に特異的に存在する細胞表面に対する抗体医療，③化学療法耐性の癌幹細胞を変化させ，化学療法の感受性をあげる，などが戦略として考えられる．たとえば，肝癌幹細胞を叩く治療法としてはCD133の抗体医療も考えられるが，先に記したように，CD133なども肝癌以外の慢性肝炎でも染色され，特異的に認識する細胞表面マーカーはない．一方で，最近オンコスタチンM（OSM）が，EpCAM陽性肝癌幹細胞を分化させることで，5-フルオロウラシル（FU）などの感受性をあげた報告も，今後注目すべきアプローチである[35]．また，癌幹細胞を維持するcancer nicheに制御機構の解析も進んでおり，cancer nicheの制御するサイトカインなどのシグナルも明らかになっており，今後はニッチ制御に注目した治療法の開発が進むと期待される．またmiRNAを導入して癌を治すという戦略も考えられる（**図❼**）．現在のところ，各種ウイルス感染との関係についてはまったく検討できていないHedgehog（Hh）シグナルが，



図❼ 癌幹細胞の制御分子

ウイルス感染によって制御される可能性も明らかになっている．今後はHBV，HCV感染と癌幹細胞，EMTとの関係についても検討していく必要がある[36]．

おわりに

本稿においては，肝癌幹細胞について最新の内容を紹介した．誌面の関係で，多くのすぐれた論文をすべて紹介できないことをここに陳謝いたします．肝癌幹細胞の理解は，より効果的な肝癌の新たな治療法の開発につながると考えられる．

【謝辞】

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Editorial

Fish model leads to new findings in liver disease

See article in *Hepatology Research* **39**: 633–644
Liver development: lessons from knockout mice and mutant fish
Takashi Nakamura, Hiroshi Nishina

In liver research, rodent models have primarily been used to identify disease mechanisms, diagnostic criteria and therapies. Tissue-specific knockout systems have also recently been developed. On the other hand, small fish such as zebrafish (*Danio rerio*)[1] and medaka (*Oryzia laptipes*) have been applied in new research models.[2,3] Medaka and zebrafish compare favorably to rodents as experimental animals for drug screening because medaka and zebrafish have a high reproductive rate, mature rapidly and cost little in terms of rearing space and daily maintenance due to their small size.

In a previous issue of the Journal, Nakamura *et al.* reported a comparative analysis of mice and medaka with regard to liver development findings.[2] In the field of liver development, mouse models were analyzed using a reverse genetic approach to identify phenotypic changes using specific gene targets.[2] Various genes, including *BMP4*, *Hhex*, *Xbp1*, *NF-κβ* and *c-jun*, were found to be important in the generation and differentiation of hepatoblasts, and new monoclonal antibodies have been found to be useful in monitoring lineage commitment during hepatocyte differentiation. Liv8/CD44 are particularly useful markers in understanding hepatoblast differentiation.[4–6]

On the other hand, medaka could be applied to forward genetic screening using N-acethyl-N-nitrosourea (ENU) treatment.[2,3] Previous forward ENU screening in Japan has established five groups of medaka liver mutants, while 19 medaka liver mutants have been established and their mutant genes are now being analyzed. In addition, 15 types of zebrafish liver mutants have been established.[1] The merit of forward genetic screening is powerful because forward screening is an unbiased approach to identify genes essential for the process of liver development.[1] In zebrafish and medaka, specific gene analysis for early development through morpholine knockdown has been shown to be effective.[7] TILLING[8] and zinc finger nucleases have recently been developed to find target gene mutants in zebrafish and medaka.[9]

We believe that new systems for drug screening using medaka and zebrafish will greatly assist in the search for new candidate drugs. Previous research using medaka and zebrafish has focused on developmental biology, but there remains an urgent need for research concerning the mechanisms and treatment of liver disease. In Japan, the history of medaka research began with a hepatocellular carcinoma model developed in the 1980s.[10] Disease models such as liver tumors in zebrafish combined with ultrasonography[11] and a non-alcoholic steatohepatitis (NASH) medaka model[12] have also recently been developed, and these models will be useful for drug screening.

We have proposed two drug screen methods using medaka and zebrafish. As shown in Figure 1(a), medaka and zebrafish liver mutants were used to screen new candidate compounds to reduce the deleterious effects of mutant genes in liver mutants. Embryos of liver mutants were bred on culture plates with various chemical compounds. Any specific chemical compounds that allow liver mutants to grow normally would thus be candidates to compensate for the effects of mutant genes. This drug screening system is specific for identifying candidate drugs for liver development. An alternative system is shown in Figure 1(b); wild-type and specific mutants showing liver disease,[3] and medaka and zebrafish with specific promoter transgenes are bred and raised on specific diets, such as high-fat, choline-deficient or N-nitrodiethylamine (DEN)-containing diets. Combination studies using liver mutants and TG

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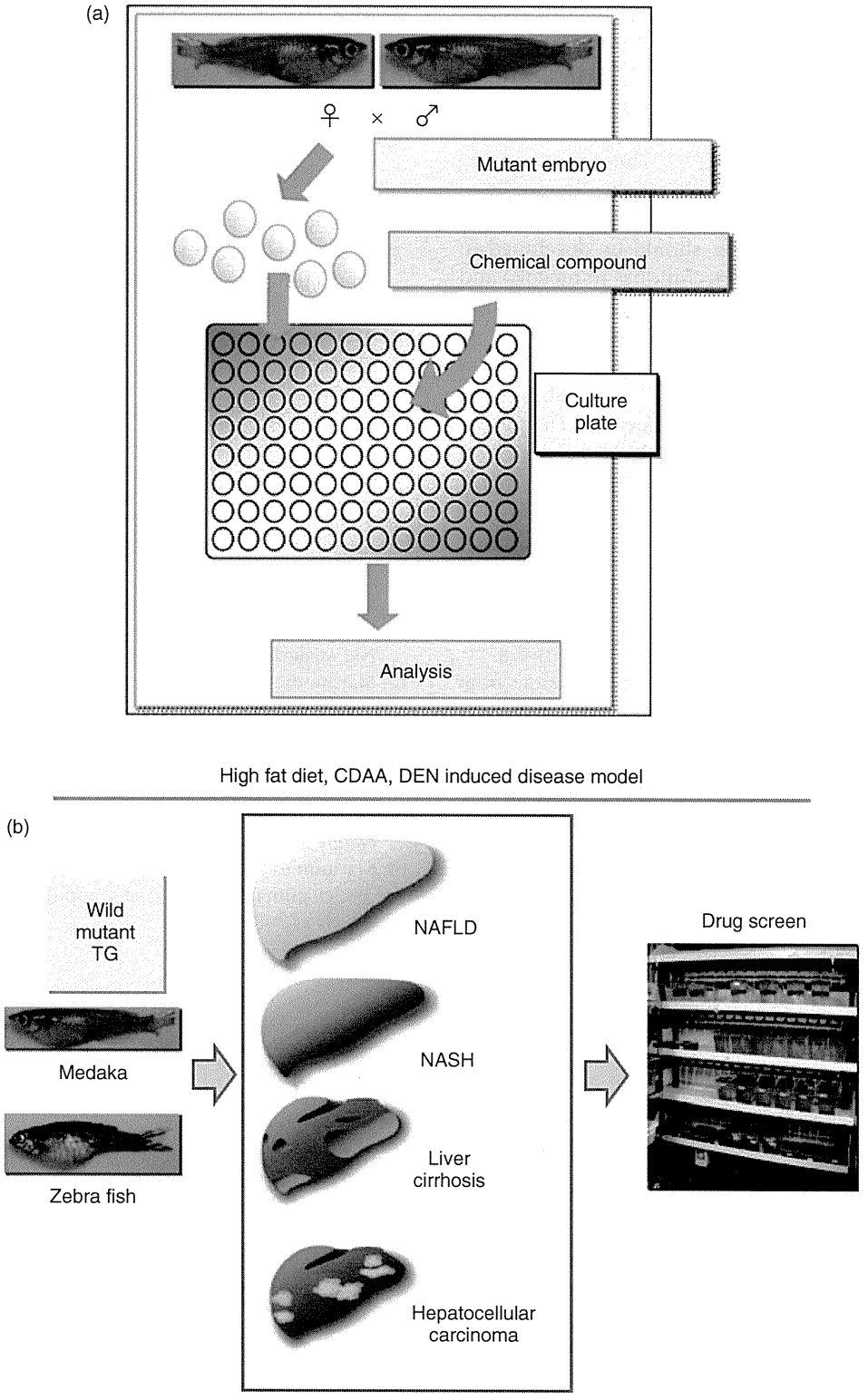

Figure 1 Drug screening using medaka and zebrafish. NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

fish with specific diets are thus useful. Using this system, candidate drugs for NASH, liver cirrhosis and hepatocellular carcinoma will be developed. Sequencing of the medaka and zebrafish genome has been completed and techniques for producing transgenic and knockout animals have been established.[1,13,14] Thus, an increasing number of genetic mechanisms can now be analyzed more efficiently.

At present, new approaches should be developed in order to incorporate the new techniques in medaka and zebrafish research. Fish models will readily allow new findings to be translated into clinical research.

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Delayed-Onset Caspase-Dependent Massive Hepatocyte Apoptosis upon Fas Activation in Bak/Bax-Deficient Mice

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The proapoptotic Bcl-2 family proteins Bak and Bax serve as an essential gateway to the mitochondrial pathway of apoptosis. When activated by BH3-only proteins, Bak/Bax triggers mitochondrial outer membrane permeabilization leading to release of cytochrome c followed by activation of initiator and then effector caspases to dismantle the cells. Hepatocytes are generally considered to be type II cells because, upon Fas stimulation, they are reported to require the BH3-only protein Bid to undergo apoptosis. However, the significance of Bak and Bax in the liver is unclear. To address this issue, we generated hepatocyte-specific Bak/Bax double knockout mice and administered Jo2 agonistic anti-Fas antibody or recombinant Fas ligand to them. Fas-induced rapid fulminant hepatocyte apoptosis was partially ameliorated in Bak knockout mice but not in Bax knockout mice, and was completely abolished in double knockout mice 3 hours after Jo2 injection. Importantly, at 6 hours, double knockout mice displayed severe liver injury associated with repression of XIAP, activation of caspase-3/7 and oligonucleosomal DNA breaks in the liver, without evidence of mitochondrial disruption or cytochrome c–dependent caspase-9 activation. This liver injury was not ameliorated in a cyclophilin D knockout background nor by administration of necrostatin-1, but was completely inhibited by administration of a caspase inhibitor after Bid cleavage. Conclusion: Whereas either Bak or Bax is critically required for rapid execution of Fas-mediated massive apoptosis in the liver, delayed onset of mitochondria-independent, caspase-dependent apoptosis develops even in the absence of both. The present study unveils an extrinsic pathway of apoptosis, like that in type I cells, which serves as a backup system even in type II cells. (HEPATOLOGY **2011;54:240-251**)

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Fas, also called APO-1 and CD95, is one of the death receptors that are potent inducers of apoptosis and constitutively expressed by every cell type in the liver.[1] Dysregulation of Fas-mediated apoptosis is involved in several liver diseases.[2] In the liver of patients with chronic hepatitis C, Fas is overexpressed in correlation with the degree of hepatitis, and Fas ligand can be detected in liver-infiltrating mononuclear cells.[3,4] Fas is also strongly expressed in the livers of patients with chronic hepatitis B, autoimmune hepatitis, and nonalcoholic steatohepatitis.[4,5] Moreover, in the liver of patients with fulminant hepatitis, Fas is up-regulated with strong detection of Fas ligand.[6] In mice, injection of Jo2 agonistic anti-Fas antibody leads

Abbreviations: ALT, alanine aminotransferase; CypD, cyclophilin D; DISC, death-inducing signaling complex; DKO, double knockout; DMSO, dimethylsulfoxide; IAP, inhibition of apoptosis protein; KO, knockout; PARP, poly(adenosine diphosphate ribose) polymerase; RIP, receptor-interacting protein; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; WT, wild-type.

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**These authors contributed equally to this work.*

to massive hepatocyte apoptosis and lethality, suggesting that the hepatocyte is one of the most sensitive cell types to Fas stimulation.[7] This model is considered to at least partly mimic human fulminant liver failure.

Fas, upon ligation by Fas ligand, activates caspase-8 through the recruitment of Fas-associated protein with a death domain and formation of the death-inducing signaling complex (DISC).[1,2] Whereas activated caspase-8 directly activates effector caspases such as caspase-3 and caspase-7 through the so-called extrinsic pathway, leading to apoptosis in type I cells, it activates caspase-3/7 through the mitochondrial pathway in type II cells. In type II cells, activated caspase-8 cleaves the BH3-only protein Bid into its truncated form, which in turn directly or indirectly activates and homo-oligomerizes Bak and/or Bax to form pores at the mitochondrial outer membrane, leading to the release of cytochrome c. After being released, cytochrome c assembles with Apaf-1 to form apoptosomes which promote self-cleavage of procaspase-9 followed by activation of caspase-3/7 to cleave a variety of cellular substrates such as poly(adenosine diphosphate ribose) polymerase (PARP) and finally to execute apoptosis.[8,9] Hepatocytes are considered to be typical type II cells, because Bid knockout (KO) mice were reported to be resistant to hepatocyte apoptosis upon Fas activation.[10,11] Although Bak and Bax are crucial gateways to apoptosis of the mitochondrial pathway, little information is available about their significance in hepatocyte apoptosis because most traditional Bak/Bax double knockout (DKO) mice ($bak^{-/-}$ $bax^{-/-}$) die perinatally.[12]

In the present study, we tried to address this issue by generating hepatocyte-specific Bak/Bax DKO mice. We demonstrate that either Bak or Bax is required and sufficient to induce Fas-mediated early-onset hepatocyte apoptosis and lethal liver injury. Importantly, even if deficient in both Bak and Bax, Bak/Bax DKO mice still develop delayed-onset caspase-dependent massive hepatocyte apoptosis, suggesting that the mitochondria-independent pathway of apoptosis, as observed in type I cells, works as a backup system when the mitochondrial pathway of apoptosis in the liver is absent. This study is the first to demonstrate the significant but limited role of Bak and Bax in executing Fas-induced apoptosis in the liver.

Materials and Methods

Mice. Heterozygous Alb-Cre transgenic mice expressing Cre recombinase gene under the promoter of the albumin gene were described.[13] We purchased Bak KO mice ($bak^{-/-}$), Bax KO mice ($bax^{-/-}$), and Bak KO mice carrying the *bax* gene flanked by 2 *lox*P sites ($bak^{-/-}$ $bax^{flox/flox}$) from the Jackson Laboratory (Bar Harbor, ME). Traditional cyclophilin D (CypD) KO mice have been described.[14] All mice strains that we used were created from a mixed background (C57BL/6 and 129). We generated hepatocyte-specific Bak/Bax DKO mice ($bak^{-/-}$ $bax^{flox/flox}$ *Alb-Cre*) or hepatocyte-specific CypD/Bak/Bax triple KO mice ($cypd^{-/-}$ $bak^{-/-}$ $bax^{flox/flox}$ *Alb-Cre*) by mating the strains. Mice were injected intraperitoneally with 1.5 or 0.5 mg/kg Jo2 anti-Fas antibody (BD Bioscience, Franklin Lakes, NJ) or intravenously with 0.25 mg/kg recombinant Fas ligand (Alexis Biochemicals, San Diego, CA) cross-linked with 0.5 mg/kg anti-Flag M2 antibody (Sigma-Aldrich, St. Louis, MO) to induce apoptosis. In some experiments, mice were intraperitoneally injected with 2 mg/kg necrostatin-1 (Sigma-Aldrich) or 40 mg/kg Q-VD-Oph (R&D Systems, Minneapolis, MN). They were maintained in a specific pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School.

Apoptosis Assay. Measurement of serum alanine aminotransferase (ALT) levels, hematoxylin and eosin staining, and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) of liver sections have been described.[15] Analysis of cytochrome c release from isolated mitochondria have also been described.[16] To detect DNA fragmentation, 1.5 μg DNA extracted from 30 mg liver tissue by Maxwell16 (Promega, Madison, WI) was incubated with 0.5 μg RNase A (Qiagen, Tokyo, Japan) and separated by way of electrophoresis on a 1.5% agarose gel.

Western Blot Analysis. For western immunoblotting, the following antibodies were used: anti–full-length Bid, anti–Cox IV, anti–cleaved caspase-3, anti–caspase-7, anti–caspase-8, anti–caspase-9, anti-PARP, anti-Bax, anti-cIAP1, and anti-XIAP antibodies were

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obtained from Cell Signaling Technology (Beverly, MA); anti-Bax and anti-cIAP2 antibodies were obtained from Millipore (Billerica, MA); anti-Bid antibody, which detects truncated Bid, was generously provided by Xiao-Ming Yin (Indiana University School of Medicine, Indianapolis, IN)[17]; and anti–β-actin antibody was obtained from Sigma-Aldrich. For isolation of the mitochondria-rich fraction, a Mitochondrial Isolation Kit (Thermo Scientific, Rockford, IL) was used. The isolation of hepatocytes from whole liver has been described.[13]

Detection of Bax Oligomerization. Liver tissue was lysed with HCN buffer (25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 300 mM NaCl, 2% CHAPS, protease inhibitor cocktail, phosphatase inhibitor cocktail, 100 μM BOC-Asp(OMe)CH2F [MP Biomedicals, Solon, OH]; pH 7.5). After the liver lysate was sonicated and centrifuged, the supernatant was collected and the concentration was adjusted. For cross-linking, 100 μL of the lysate was incubated with 5 μL 100 mM bis(maleimido)hexane (Thermo Scientific) and 5 μL 100 mM BS^3 (Thermo Scientific) for 30 minutes at room temperature as described.[18] After quenching the cross-linkers by way of incubation with 12 μL 1 M Tris-HCl (pH 7.5) for 15 minutes at room temperature, the lysate was boiled with sample buffer followed by western blot analysis for Bax.

Electron Microscopy. Livers were fixed by perfusion of phosphate-buffered saline with 2.5% glutaraldehyde solution buffered at pH 7.4 with 0.1 M Millonig's phosphate, postfixed in 1% osmium tetroxide solution at 4°C for 1 hour, dehydrated in graded concentrations of ethanol, and embedded in Quetol 812 epoxy resin (Nisshin EM, Tokyo, Japan). Ultrathin sections (80 nm) cut on ultramicrotome were stained with uranyl acetate and lead citrate and examined with an H-7650 electron microscope (Hitachi Ltd., Tokyo, Japan) at 80 kV.

Statistical Analysis. Data are presented as the mean $\pm$ SE. Differences between two groups were determined using the Mann-Whitney U test for unpaired observations. The survival curves were estimated using the Kaplan-Meier method and were tested by way of log-rank test. $P < 0.05$ was considered statistically significant.

Results

Bak Deficiency Partially Ameliorates Fas-Induced Hepatocellular Apoptosis but Fails to Prevent Animal Death. First, to examine the significance of Bak in hepatocellular apoptosis induced by Fas stimulation, Bak KO mice ($bak^{-/-}$) and wild-type (WT) littermates ($bak^{+/+}$) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody and analyzed 3 hours later. Consistent with previous reports,[10,19] WT mice showed severe elevation of serum ALT levels with massive hepatocellular apoptosis (Fig. 1A,B). Bak KO mice also developed liver injury, but the levels of serum ALT and the number of TUNEL-positive hepatocytes were significantly lower in Bak KO mice than in WT mice (Fig. 1A-C). Western blotting for cleaved caspase-3, caspase-7, and PARP revealed that activation of effector caspases were partially inhibited in KO livers compared with WT livers (Fig. 1D). Cleavage of procaspase-9, which is initiated by mitochondrial release of cytochrome c, was also suppressed in Bak KO livers compared with WT liver (Fig. 1D). The cleaved form of caspase-8, a direct downstream target of Fas activation, was detected in both mice, but its levels were reduced in Bak KO mice compared with WT mice (Fig. 1D). This reduction may be explained by the lesser activation of caspase-3/7, because it has been reported that caspase-3/7 could activate caspase-8 through an amplification loop during apoptosis.[20] Collectively, these findings demonstrated that Bak deficiency partially ameliorated Fas-induced hepatocellular apoptosis associated with reduced cleavage of caspase-9, caspase-3/7, and PARP. We then compared survival of mice after Jo2 injection but found that Bak KO mice also rapidly died with kinetics similar to those of WT mice, suggesting that partial amelioration of hepatocellular apoptosis induced by Bak deficiency did not lead to survival benefit under our experimental conditions (Fig. 1E). Because Bax residing in the cytosol moves to the mitochondria upon activation, where it undergoes oligomerization,[21] we analyzed its translocation and oligomerization in the liver at 3 hours after Jo2 injection. Western blot analysis revealed that the levels of Bax expression clearly increased in the mitochondrial fraction in both WT livers and Bak KO livers (Fig. 1F). Signals for the Bax dimer were also detected in both livers (Fig. 1F). These findings indicate that Bax is also activated after Fas stimulation, raising the possibility of its involvement in hepatocellular apoptosis.

Bax Deficiency Fails to Ameliorate Fas-Induced Hepatocellular Apoptosis. Next, to examine the significance of Bax in hepatocellular apoptosis induced by Fas stimulation, Bax KO mice ($bax^{-/-}$) and WT littermates ($bax^{+/+}$) were injected with Jo2 and examined 3 hours later. There was no significant difference in the levels of serum ALT or the number of TUNEL-positive hepatocytes between the two groups (Fig. 2A-C), which is consistent with a previous report.[22] The levels of the cleaved forms of caspase-8, -9, -3, -7, and

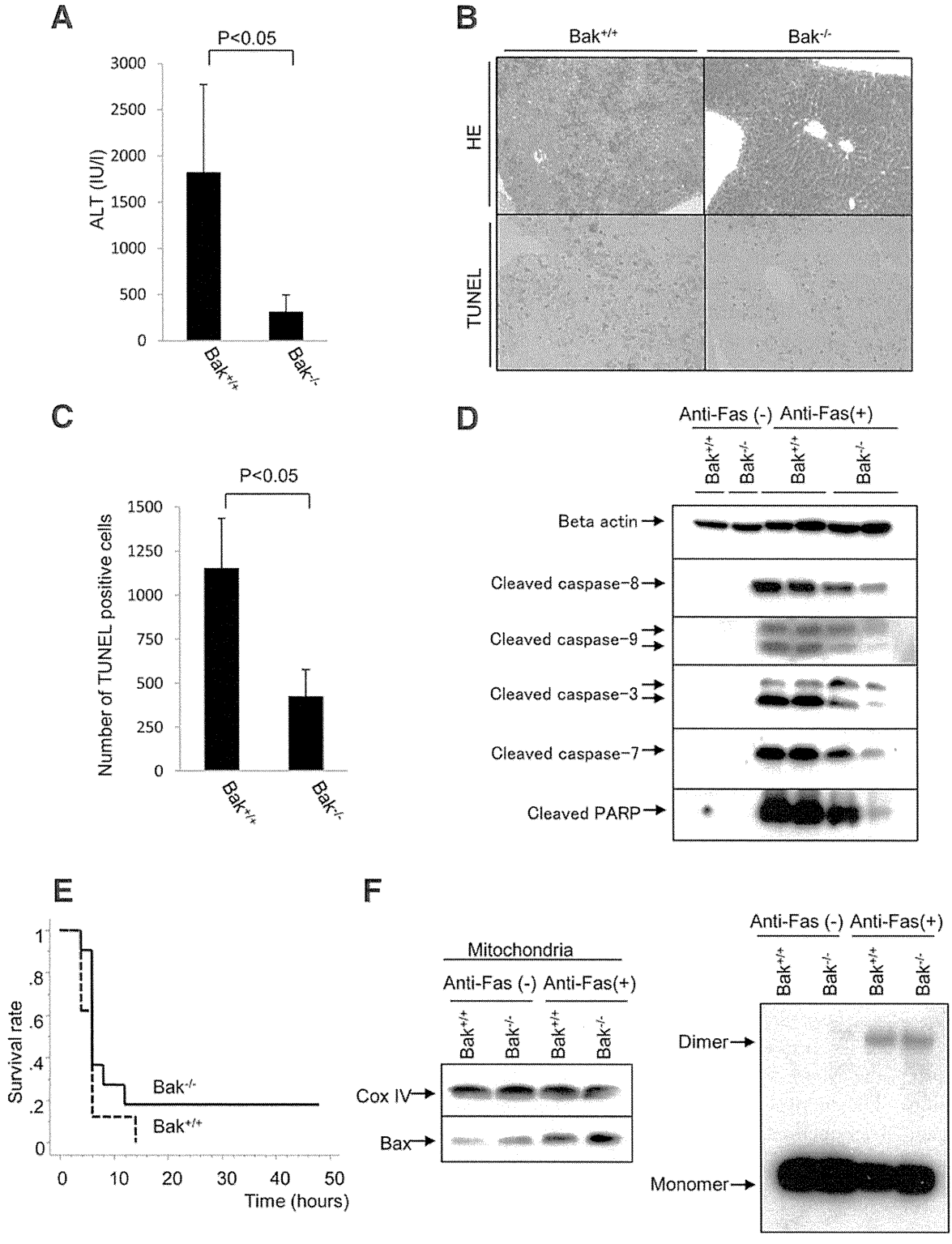

Fig. 1. Bak KO mice are partially resistant to Fas-induced hepatocellular apoptosis. Bak KO mice ($Bak^{-/-}$) or control WT littermates ($Bak^{+/+}$) were analyzed at 3 hours after intraperitoneal injection of 1.5 mg/kg Jo2 anti-Fas antibody. (A) Serum ALT levels (n = 10 or 11, respectively). (B) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (C) Number of TUNEL-positive cells (n = 8 or 9, respectively). (D) Western blot analysis for the expressions of cleaved caspase-8, 9, -3, -7 and PARP. (E) Bak KO mice or control WT littermates were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody (n = 8 or 11, respectively). Survival rates after Jo2 injection are shown. (F) Bak KO mice or control WT littermates were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg) or vehicle. Left: Western blot analysis of the mitochondrial fraction of the liver for the expression of Bax. Right: Western blot analysis for the expression of Bax monomer and dimer in the liver.

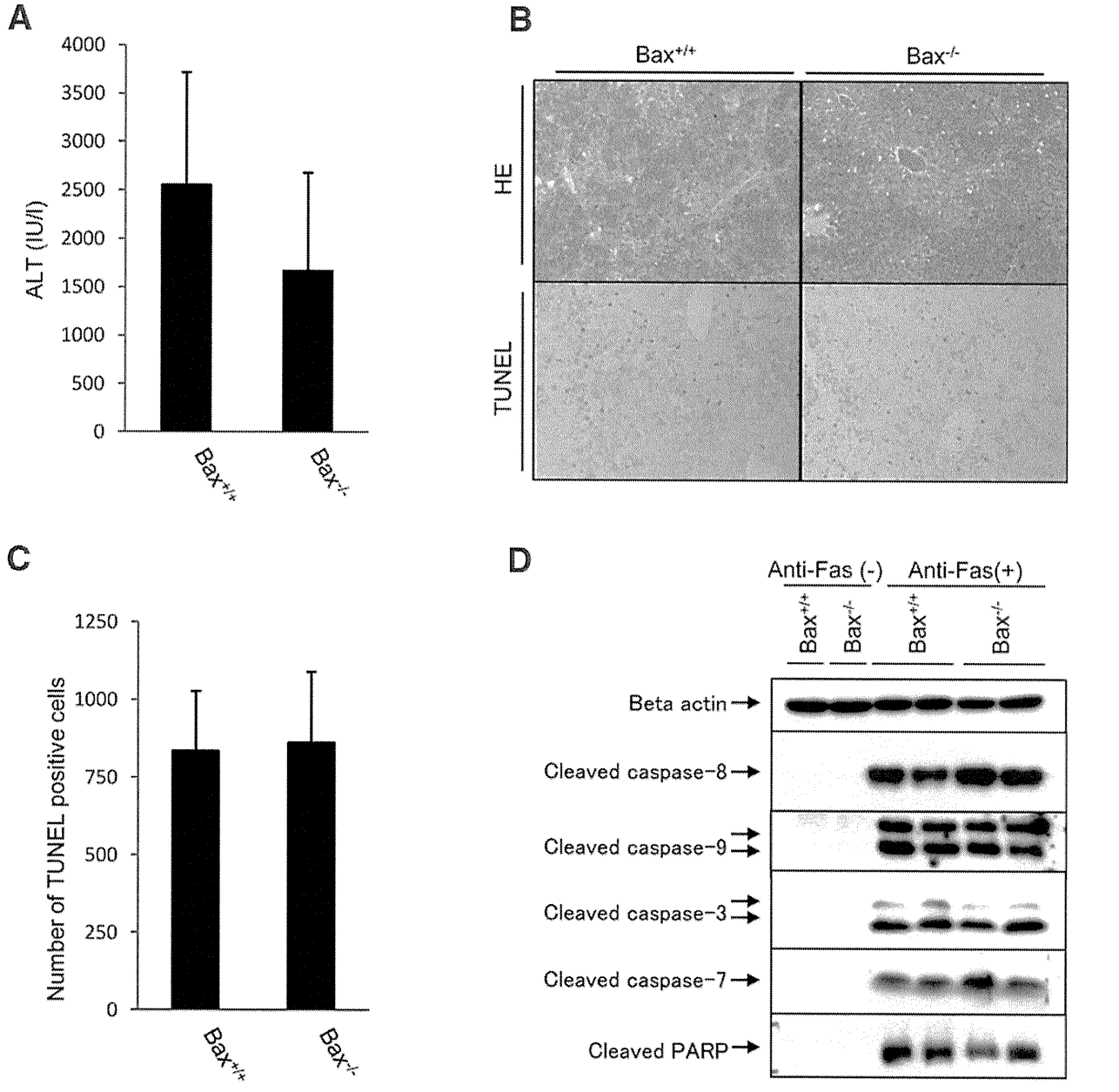

Fig. 2. Bax KO mice are not resistant to Fas-induced hepatocellular apoptosis. Bax KO mice ($Bax^{-/-}$) or control WT littermates ($Bax^{+/+}$) were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). (A) Serum ALT levels (n = 11 per group). (B) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (C) Number of TUNEL-positive cells (n = 8 per group). (D) Western blot analysis for the expressions of cleaved caspase-8, -9, -3, -7, and PARP.

PARP in Bax KO livers did not differ from those of WT livers (Fig. 2D). These findings demonstrate that, in contrast to Bak deficiency, Bax deficiency was not able to inhibit Fas-induced hepatocellular apoptosis.

Bax Deficiency Completely Blocks Fas-Induced Early-Onset Hepatocellular Apoptosis in a Bak-Deficient Background. To examine the impact of Bax in a Bak-deficient background, hepatocyte-specific Bak/Bax DKO mice (*bak*$^{-/-}$ *bax*$^{flox/flox}$ *Alb-Cre*) and Bak KO mice (*bak*$^{-/-}$ *bax*$^{flox/flox}$), which served as control littermates of this mating, were injected with Jo2 and analyzed 3 hours later. We confirmed the hepatocyte-specific defects of Bax protein in Bak/Bax DKO mice by way of western blot analysis (Fig. 3A). The serum ALT levels of Bak/Bax DKO mice were in the normal range and were significantly lower than those of Bak KO mice (Fig. 3B). Liver histology and TUNEL staining did not show evidence of hepatocyte apoptosis in Bak/Bax DKO livers, in contrast to Bak KO livers (Fig. 3C,D). Taken together, these results indicate that Bak and Bax are basically redundant molecules for execution of hepatocellular apoptosis induced by Fas

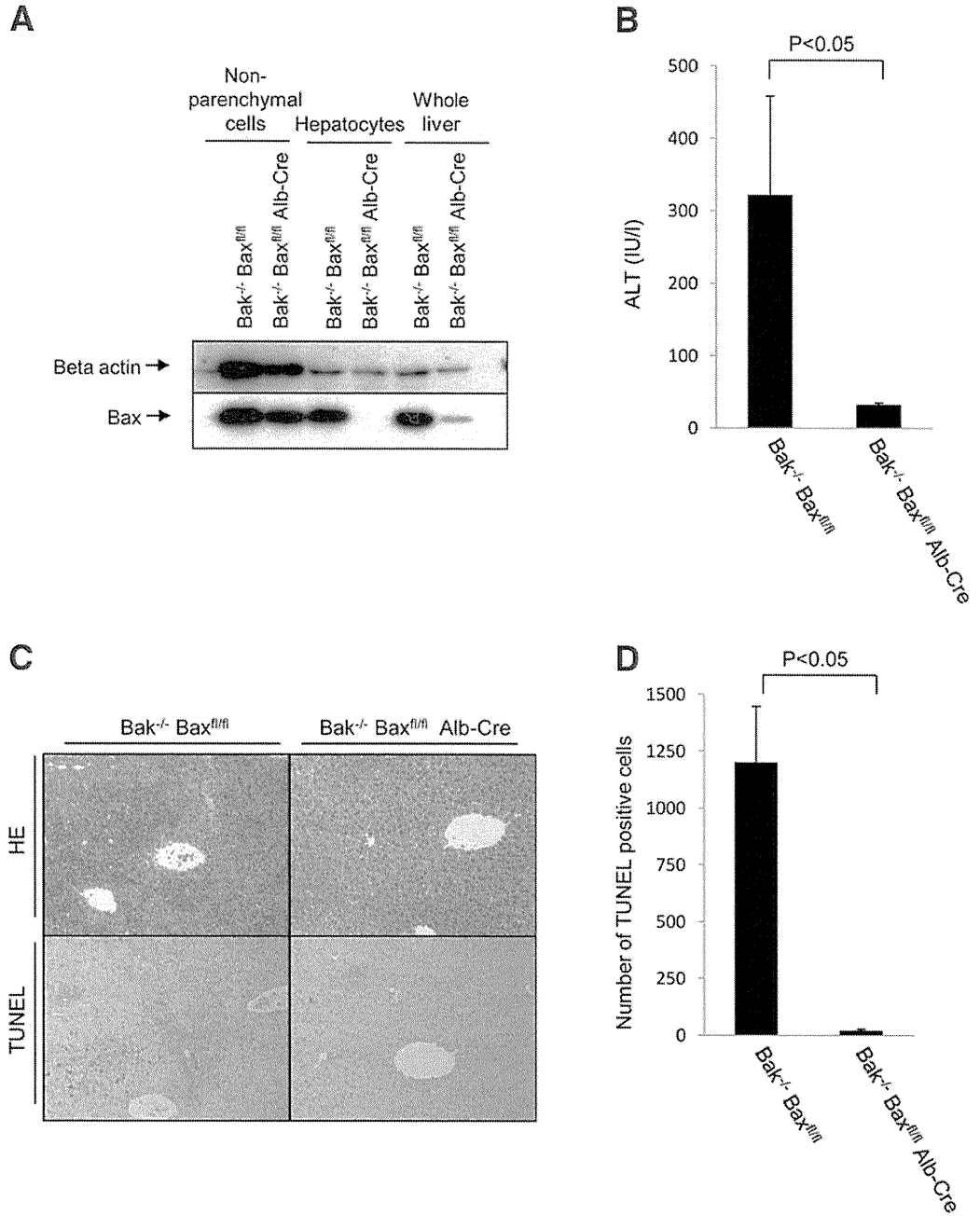

Fig. 3. Bak/Bax DKO mice are fully resistant to Fas-induced hepatocellular apoptosis in early phase. (A) Western blot analysis of the indicated fraction of the liver for the expressions of Bax. (B-D) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). (B) Serum ALT levels (n = 10 per group). (C) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (D) Number of TUNEL-positive cells (n = 9 per group).

activation, although the former appears to be clearly required for full-blown apoptosis in vivo.

Fas Stimulation Leads to Late-Onset Hepatocellular Death Even in Bak/Bax Deficiency with Moderate Caspase-3/7 Activation Without Mitochondrial Disruption. To examine whether the inhibition of Fas-induced rapid liver injury in Bak/Bax deficiency is a durable effect, we analyzed the survival rate after Jo2 injection. The survival rate of Bak/Bax DKO mice was significantly higher than that of Bak KO mice, but approximately half of the Bak/Bax DKO mice died within 12 hours (Fig. 4A). To examine the cause of this late-onset lethality, we analyzed the serum ALT levels and liver tissue 6 hours after Jo2 injection. Unexpectedly, the serum ALT levels were highly elevated in Bak/Bax DKO mice (Fig. 4B). Liver histology revealed many hepatocytes with cellular shrinkage and scattered regions of sinusoidal hemorrhage (Fig. 4C), indicating that Bak/Bax DKO mice still developed severe liver injury at this time point. TUNEL staining

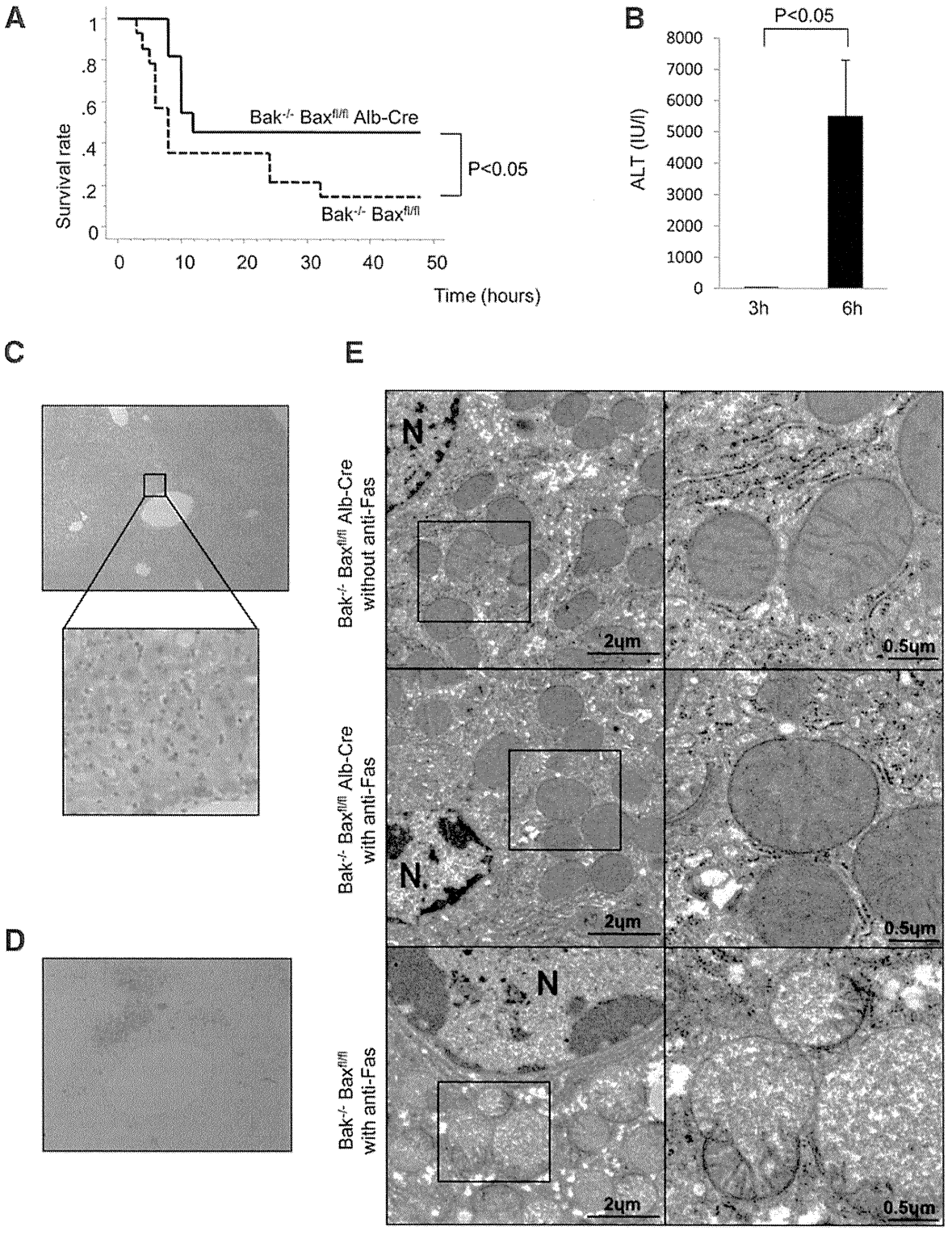

Fig. 4. Bak/Bax DKO mice develop late-onset severe liver injury upon Fas stimulation. Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody. (A) Survival rate after Jo2 injection (n = 11 per group). (B) Serum ALT levels of Bak/Bax DKO mice. (C, D) Hematoxylin and eosin (C) and TUNEL (D) staining of the liver sections of Bak/Bax DKO mice 6 hours after Jo2 injection. Representative photomicrographs are shown. (E) Representative electron microscopy photomicrographs of the livers of Bak/Bax DKO mice before and 6 hours after Jo2 anti-Fas injection (1.5 mg/kg) and control Bak KO mice 2 hours after Jo2 anti-Fas injection (1.5 mg/kg). Right panels are enlarged images of the square area of each left panel. N, nucleus.

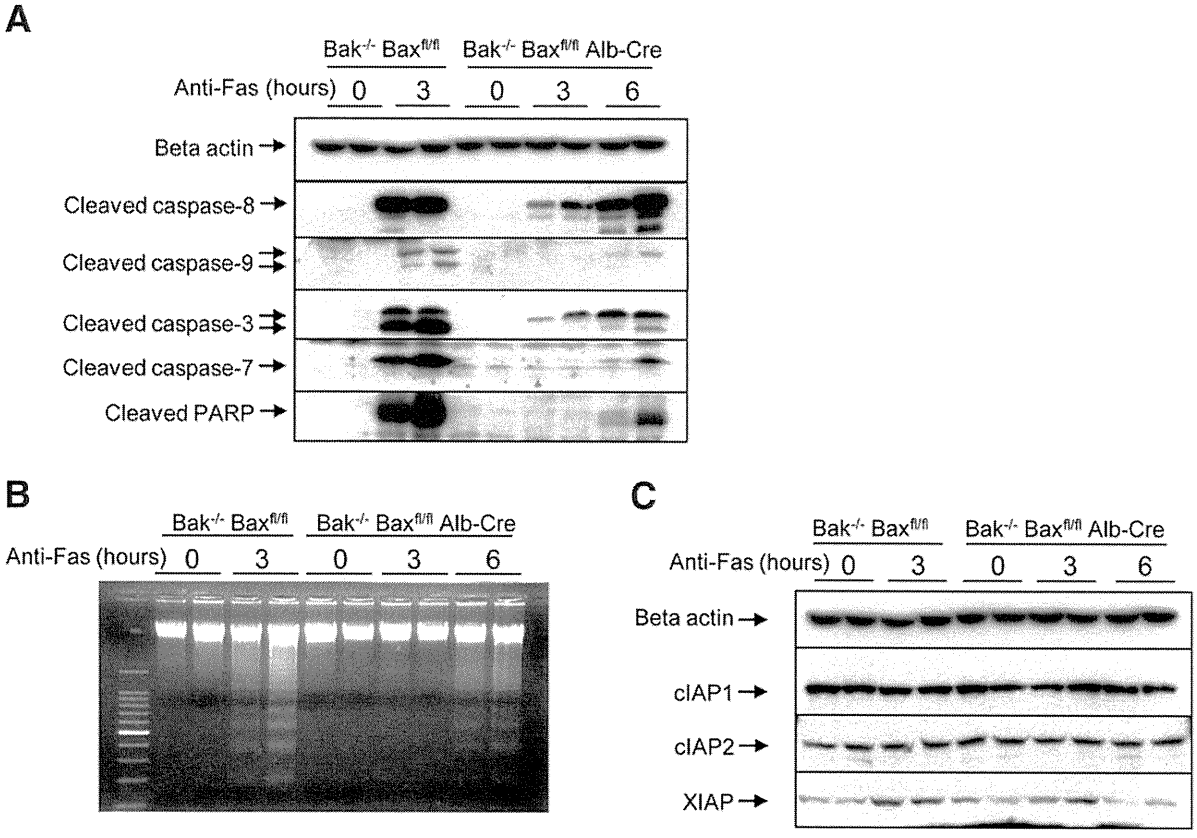

Fig. 5. Fas-mediated hepatocellular death in Bak/Bax DKO mice is associated with caspase-3/7 activation and oligonucleosomal DNA breaks. Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were intraperitoneally injected with Jo2 anti-Fas antibody (1.5 mg/kg). (A) Western blot analysis for expression of cleaved caspase-8, -9, -3, -7, and PARP. (B) DNA laddering of the liver. (C) Western blot analysis for expression of IAP family proteins.

revealed many TUNEL-positive hepatocytes in the liver sections. Of importance, electron microscopic analysis revealed mitochondrial alterations (such as disruption of the membrane and herniation of the matrix) in hepatocytes of Bak KO mice but not in hepatocytes of Bak/Bax DKO mice with chromatin condensation (Fig. 4E). Because some reports showed that hepatocytes act like type I cells with a high dose of Jo2 anti-Fas antibody and that anti-Fas antibody does not always reliably mimic the action of the physiological Fas ligand,[23,24] we also injected 0.5 mg/kg Jo2 or recombinant Fas ligand into Bak/Bax DKO mice. Similarly, both injected mice showed severe elevation of serum ALT levels and severe hepatitis with many TUNEL-positive cells at 6 hours (Supporting Figs. 1 and 2).

To examine the kinetics of caspase activation and apoptosis in the liver after Jo2 administration, we performed western blot analysis for caspase activation and agarose gel electrophoresis for DNA laddering. All signals for cleaved forms of caspase-3, caspase-7, and PARP in the liver were clearly detected at 6 hours in Bak/Bax DKO mice, although they were weaker than those at 3 hours in control Bak KO littermates (Fig. 5A). Regarding the cleaved form of caspase-9, two bands were detected at 3 hours in Bak KO liver, but not in Bak/Bax DKO liver. Previous research established that procaspase-9 has two sites for cleavage upon activation: both Asp353 and Asp368 sites are autoprocessed by caspase-9 activation after cytochrome c release, whereas the Asp368 site is preferentially processed over the Asp358 site by caspase-3.[25] In our western blot analysis, the slow migrating species corresponding to the fragment cleaved at Asp368, but not the rapid migrating species corresponding to that at Asp353, was weakly detected at 6 hours in Bak/Bax DKO liver. This indicated that caspase-3–mediated cleavage of procaspase-9 takes place without evidence of cytochrome c–induced autoprocessing of procaspase-9. Agarose gel electrophoresis clearly detected oligonucleosomal DNA laddering at 6 hours in Bak/Bax DKO livers, similar to our observation at 3 hours in control Bak KO livers (Fig. 5B). Collectively, these morphological and biochemical data support the idea that hepatocellular death occurring at 6 hours in the Bak/Bax DKO liver seems to involve apoptosis.

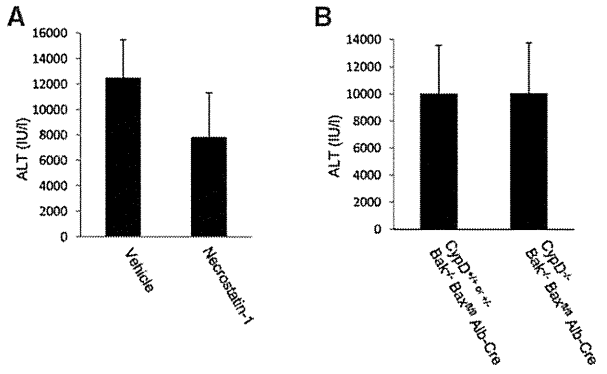

Fig. 6. Fas-induced hepatocellular death in Bak/Bax DKO mice is independent of RIP kinase and/or CypD. (A) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Cre) were intraperitoneally injected with 2 mg/kg necrostatin-1 in vehicle containing 0.2% dimethylsulfoxide or vehicle alone at 2 hours after injection of 1.5 mg/kg Jo2 anti-Fas antibody. Serum ALT levels at 6 hours after Jo2 injection are shown (n = 8 per group). (B) $CypD^{+/+\ or\ +/-}$ mice in a Bak/Bax-deficient background ($CypD^{+/+\ or\ +/-}$ $Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control $CypD^{-/-}$ littermates ($CypD^{-/-}$ $Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody. Serum ALT levels at 6 hours after injection are shown (n = 7 per group or 8 per group, respectively).

To examine the underlying mechanisms by which caspase-3/7 was increasingly activated from 3 to 6 hours in Bak/Bax DKO mice, we analyzed the expression of inhibition of apoptosis proteins (IAPs), which can block cleavage of procaspase-3, -7, and -9.[26] The expression levels of cIAP1 and cIAP2 were not changed in the liver after Jo2 injection (Fig. 5C, Supporting Fig. 3). In contrast, the expression levels of XIAP were up-regulated in the livers of both Bak KO and Bak/Bax DKO mice at 3 hours after Jo2 injection, as in WT mice (Fig. 5C, Supporting Fig. 3), which is consistent with previous reports.[27] However, this up-regulation disappeared from the livers of Bak/Bax DKO mice at 6 hours. Repression of XIAP overexpression might explain why weak activation of capsase-3/7 gradually increased from 3 to 6 hours in the Bak/Bax DKO liver.

Cell Death with Bak/Bax Deficiency Is Not Dependent on a Necrotic Pathway. Fas activation was reported to induce not only caspase-dependent apoptosis but also caspase-independent necrosis, which is required for receptor-interacting protein (RIP) kinase.[28] To exclude the possibility of this necrotic cell death in the Bak/Bax DKO liver, we first examined the effect of necrostatin-1, which specifically inhibits RIP kinase to protect against necrotic cell death caused by death-domain receptor stimulation.[2,29] Bak/Bax DKO mice were injected with 2 mg/kg necrostatin-1 at 2 hours after or 1 hour before Jo2 injection. The ALT levels at 6 hours after Fas stimulation were clearly elevated without a significant difference between the necrostatin-1 injection group and the vehicle injection group (Fig. 6A and Supporting Fig. 4). We next examined the effect of CypD, which is a key molecule of mitochondrial permeability transition generated by Ca^{2+} overload and/or oxidative stress leading to necrotic cell death.[14,30] We injected Jo2 into $CypD^{-/-}$ mice with a Bak/Bax-deficient background (*$cypd^{-/-}$ $bak^{-/-}$ $bax^{flox/flox}$ Alb-Cre*) or control $CypD^{+/+\ or\ +/-}$ littermates (*$cypd^{+/+\ or\ +/-}$ $bak^{-/-}$ $bax^{flox/flox}$ Alb-Cre*). The ALT levels of CypD/Bak/Bax triple KO mice upon Fas stimulation were the same as those of control mice (Fig. 6B). These results indicate that liver injury in Bak/Bax deficiency induced by Fas stimulation was not dependent on the necrotic pathway, at least that mediated by RIP kinase and/or CypD.

Late-Onset Cell Death in Bak/Bax Deficiency Is Completely Dependent on Caspase. Although cell death observed in Bak/Bax DKO mice appears to be apoptosis, the question arose of whether relatively weak caspase-3/7 activity compared with that observed in Bak KO mice is sufficient for inducing liver injury 6 hours after Fas simulation. To this end, Bak/Bax DKO mice were given 40 mg/kg Q-VD-Oph, a potent broad spectrum caspase inhibitor,[31] 2 hours after injection of Jo2. Western blot analysis revealed the existence of truncated Bid and cleaved caspase-8 in the liver 2 hours after Jo2 injection, demonstrating that caspase-8 had already been activated by this point (Fig. 7A). Administration of the caspase inhibitor at 2 hours completely blocked the elevation of serum ALT levels and hepatocellular apoptosis, as evidenced by liver histology and TUNEL staining 6 hours after Jo2 injection (Fig. 7B-D). Finally, we tried to analyze the survival rate of Bak/Bax DKO mice and control Bak KO mice when therapeutically injected with the caspase inhibitor 2 hours after Jo2 injection. None of the Bak/Bax DKO mice showed lethal liver injury upon Jo2 injection, whereas half of the Bak KO mice died from severe liver injury (Fig. 7E). These findings suggest that Fas-induced liver injury in Bak/Bax deficiency was dependent on caspase activity, which could be fully negated by the caspase inhibitor. On the other hand, caspase activation in Bak KO mice was too high to be negated by the same dose of the caspase inhibitor.

Discussion

In the present study, we demonstrate that Bak KO, but not Bax KO, provides partial resistance to Fas-induced hepatocellular apoptosis in vivo. We demonstrated previously that Bak KO mice, but not Bax KO

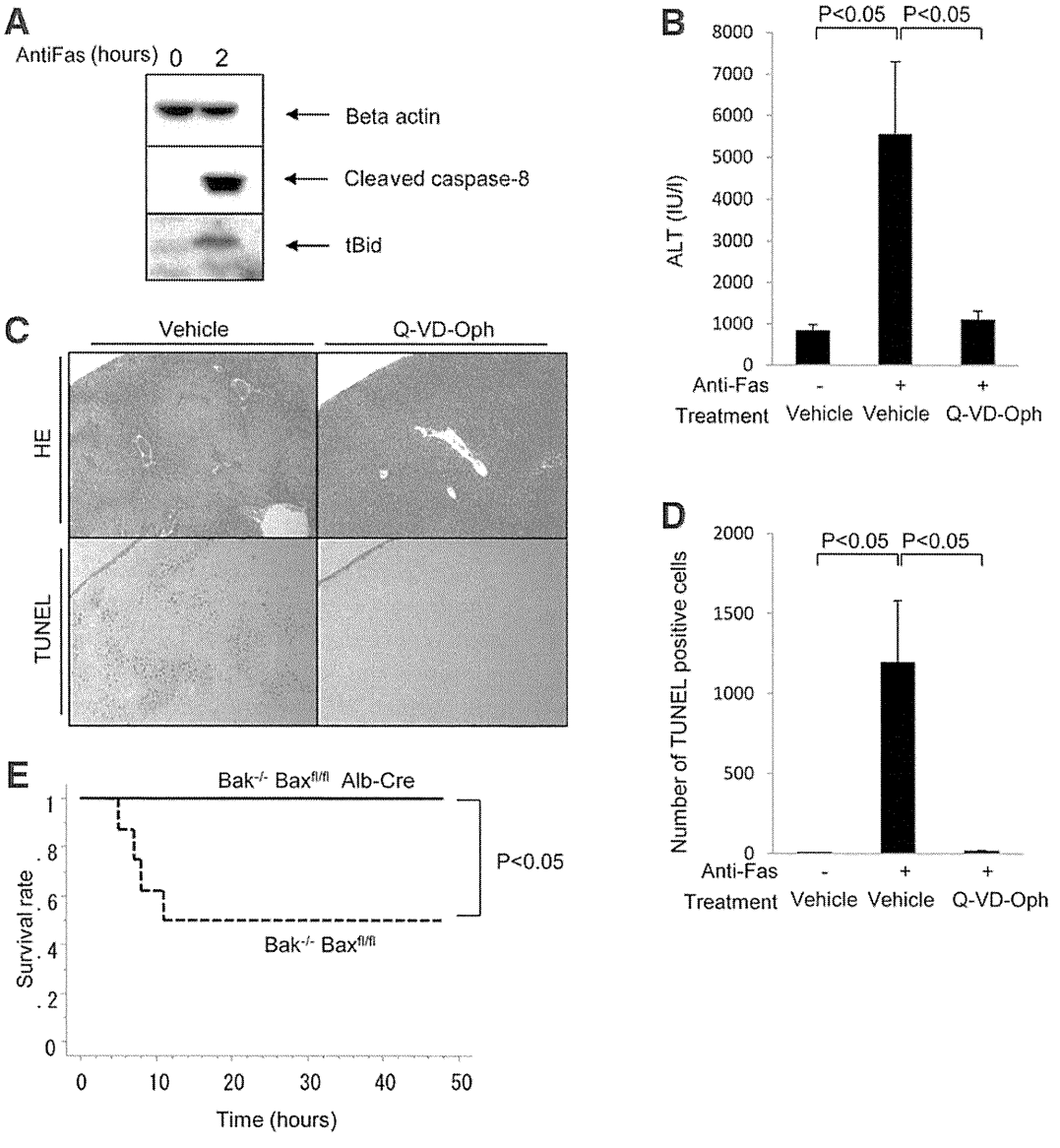

Fig. 7. Hepatocellular death in Bak/Bax DKO mice is dependent on caspase activation. (A) Bak/Bax DKO mice were analyzed before and 2 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). Western blot analysis of the liver for the expression of cleaved caspase-8 and truncated Bid (tBid). (B-D) Bak/Bax DKO mice were intraperitoneally administered 40 mg/kg Q-VD-Oph in 10 mL/kg dimethylsulfoxide (DMSO) or DMSO alone, as a vehicle, 2 hours after injection of 1.5 mg/kg Jo2 anti-Fas antibody and analyzed at 6 hours. (B) Serum ALT levels (n = 6 or 7 per group, respectively). (C) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (D) Number of TUNEL-positive cells (n = 6 or 7 per group, respectively). Because intraperitoneal injection of DMSO leads to injury at the surface layer of the liver, TUNEL positivity close to the surface layer was excluded from the cell count. (E) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were given 40 mg/kg Q-VD-Oph intraperitoneally in 10 mL/kg DMSO or DMSO alone at 2 hours after injection of 1.5 mg/kg anti-Fas antibody. The disease-free survival rate of lethal liver injury after Jo2 injection is shown (n = 8 per group).

mice, showed resistance to apoptosis induced by Bcl-xL deficiency, which depended mainly on Bid activation.[16] Research has shown that Fas induces apoptosis in hepatocytes through the Bid pathway,[10,11] and the present study also demonstrates that Bid becomes truncated in the liver upon anti-Fas injection. Therefore, truncated Bid may preferentially activate Bak rather than Bax in the liver. However, the present study also reveals that, in the absence of Bak, Bax plays an essential role in mediating the early onset of hepatocellular apoptosis. The most important finding of this study is that Bak/Bax deficiency failed to protect against the late onset of liver injury after Jo2 anti-Fas injection as well as Fas agonist injection. Wei et al.,[32] in their historical paper establishing the importance of Bak and Bax in the mitochondrial pathway of apoptosis, reported that hepatocytes were protected from Jo2-induced apoptosis in traditional Bak/Bax DKO mice ($bak^{-/-}$ $bax^{-/-}$). Because perinatal lethality occurs with most traditional Bak/Bax DKO mice,

they could only analyze three animals, which did not enable detailed analysis of cell death due to Jo2 stimulation. The present study is the first to (1) thoroughly examine the impact of Bak and Bax in the liver using conditional KO mice and (2) demonstrate that Bak/Bax deficiency can protect against Fas-induced severe injury in the early phase but not in the late phase.

The late onset of liver injury observed in Bak/Bax DKO appeared to be apoptosis based on biochemical and morphological observations, including caspase activation, oligonucleosomal DNA breaks and, most importantly, identification of cell death with caspase dependency. In addition, the well-established necrotic pathway mediated by RIP kinase and/or CypD was not involved. However, the difference from apoptosis observed in Bak KO mice was the absence of mitochondrial alteration or cytochrome c–dependent caspase-9 processing in Bak/Bax DKO mice. We also confirmed that Bak/Bax-deficient mitochondria were not capable of releasing cytochrome c in the presence of truncated Bid (Supporting Fig. 5). These data support the idea that activation of the mitochondrial pathway of apoptosis is fully dependent on either Bak or Bax even in the late phase, indicating at the same time that late onset of apoptosis takes place through an extrinsic pathway rather than the mitochondrial pathway.

Although hepatocytes are generally considered to be type II cells, recent work has shown that the requirement of the mitochondrial pathway may be overcome through changes induced by in vitro culture conditions[33,34] or the strength of Fas stimulation.[23] Schüngel et al.[23] demonstrated that hepatocytes act as type II cells with a low-dose Jo2 injection (0.5 mg/kg) and act as type I cells with an extremely high-dose Jo2 injection (5 mg/kg). This agrees with the generally accepted idea that type I cells exhibit strong activation of DISC and caspase-8, which itself is sufficient to induce apoptosis, whereas type II cells exhibit weak activation and therefore require amplification of the apoptosis signal through the mitochondrial loop. In the present study, we used 1.5 mg/kg or 0.5 mg/kg Jo2 antibody, which could be considered relatively low doses, and found that hepatocytes act like type II cells in WT mice or Bak/Bax single KO mice but act like type I cells in Bak/Bax DKO mice. The present study therefore demonstrates that hepatocytes can act as type I cells in the absence of Bak and Bax independent of the strength of DISC formation or signals from microenvironments.

The question arises of why hepatocytes can act as type I cells where the levels of DISC formation or caspase-8 activation may be insufficient to induce activation of downstream caspases. Recently, Jost et al.[27] reported a discriminating role of XIAP between type I and type II cells; in type II cells, the levels of XIAP expression increased after Fas stimulation but decreased in type I cells. In agreement with this report, XIAP expression was up-regulated at 3 hours in both Bak KO and Bak/Bax DKO livers. Interestingly, this XIAP up-regulation disappeared at 6 hours after Jo2 injection in Bak/Bax DKO mice. Because XIAP is a potent inactivator of caspase-3, -7, and -9 processing, repression of XIAP may be one reason why hepatocytes can act as type I cells at this time point.

Previous studies have reported that liver endothelial cells express Fas receptor and have suggested that apoptosis of these cells may participate in the liver damage in mice receiving Jo2 antibody, especially in the case of high-dose administration.[35] However, we did not find liver injury in the sinusoidal hemorrhage in Bak/Bax DKO mice at 3 hours after Jo2 injection, which is the time point when Bak KO mice developed it (Fig. 3C). Together with the fact that Bax, but not Bak, was active in liver nonparenchymal cells in our Bak/Bax DKO mice, as was the case in Bak KO mice (Fig. 3A), we speculate that Bak-deficient sinusoidal cells could not contribute much to liver injury at 3 hours after Jo2 injection (1.5 or 0.5 mg/kg).

Recently, a pan-caspase inhibitor was reported to reduce hepatic damage in liver transplant recipients and patients with chronic hepatitis C in clinical trials.[36,37] For treatment of fulminant liver injury, caspase inhibitors seem to be attractive drugs. However, the present study demonstrates that Fas-induced apoptotic signals could be efficiently amplified through the mitochondrial pathway, leading to high lethality even if caspase inhibitor was administered 2 hours after Jo2 injection. In contrast, administration of the same dose of the caspase inhibitor was able to fully block hepatocyte apoptosis and lethality in Bak/Bax DKO mice. From a clinical point of view, when using caspase inhibitors to prevent fulminant liver failure, concomitant inactivation of the mitochondrial amplification loop may be required.

In conclusion, the extrinsic pathway of apoptosis exists in hepatocytes and causes late onset of lethal liver failure in the absence of Bak and Bax independent of the strength of Fas ligation. This pathway could be therapeutically intervened through the use of caspase inhibitors, presumably due to low levels of DISC formation and subsequent weak activation of effector caspases in hepatocytes. The present study unveils the entire framework of the Fas-mediated signaling