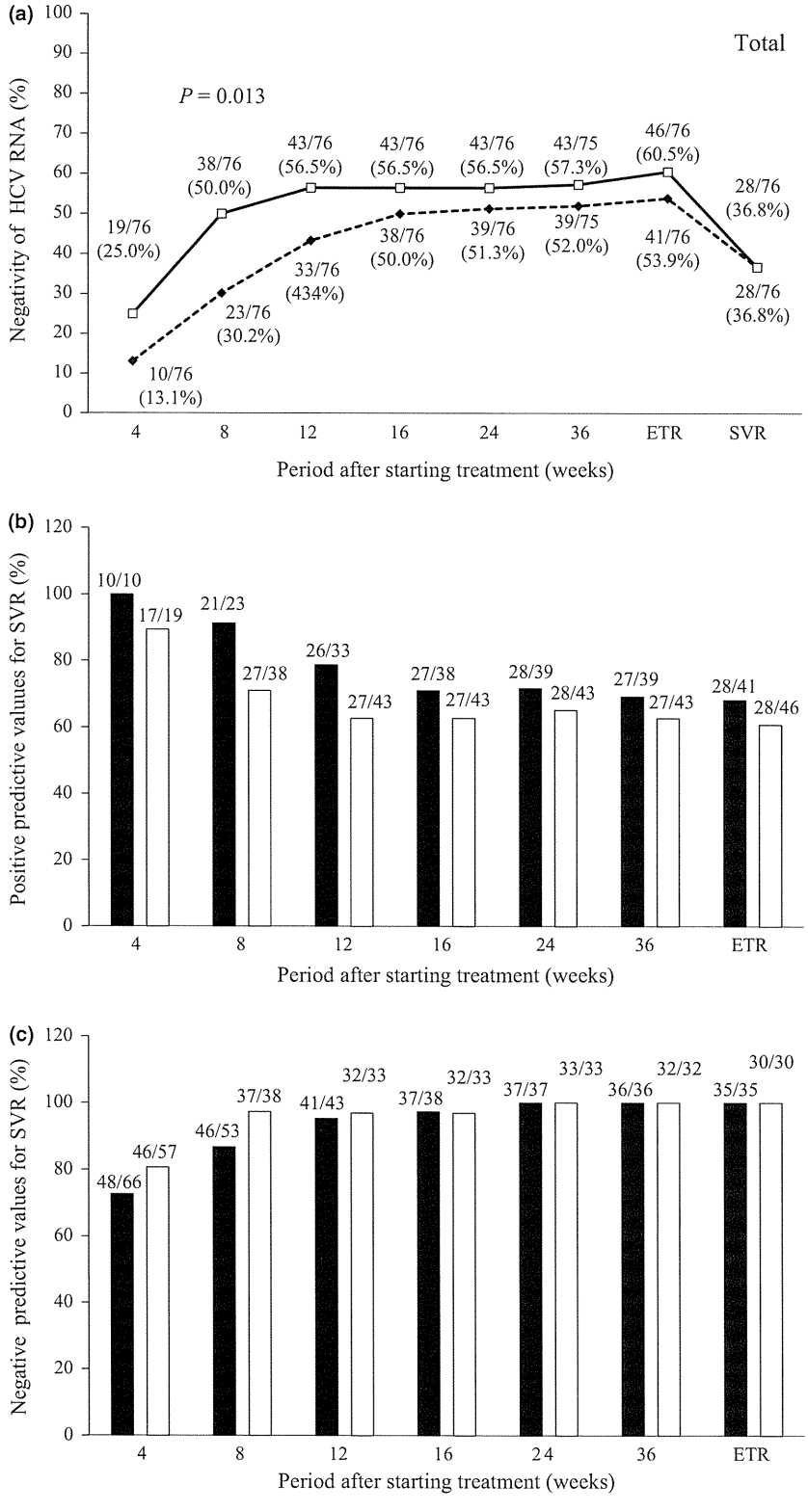

Fig. 1 (a) Proportions of patients with HCV RNA lower than the limits of detection (black diamonds) and HCV RNA <1.7 log IU/mL (white squares) by COBAS TaqMan HCV test over time in the treatment cohort. Total 76 patients were HCV RNA-positive at pretreatment baseline. (b, c) Predictive values for SVR and for non-SVR in patients with HCV RNA lower than the limits of detection (black columns) and HCV RNA <1.7 log IU/mL (white columns) by COBAS TaqMan HCV test over time in the treatment cohort. (b) Positive predictive values for SVR. (c) Negative predictive values for SVR. ETR, virological response at the end of treatment; SVR, undetectable serum HCV RNA at 24 weeks after the end of treatment; *P*-value, significant difference between two groups by chi-square test.

whether there were discrepancies between undetectable RNA and <1.7 log IU/mL. We showed that the utilization of both undetectable RNA and values ≤1.7 log IU/mL HCV RNA by COBAS TaqMan HCV test is useful and could predict SVR and non-SVR patients with greater accuracy. Our study also showed that the COBAS TaqMan HCV test is a more accurate method for evaluating EVR and RVR, although the negative predictive value was superior when ≤1.7 log IU/mL HCV RNA by COBAS TaqMan HCV test was used.

Recently, there have been reports that in HCV genotype 2/3-infected patients with a very rapid viral response (vRVR), i.e. HCV RNA below 1000 IU/ml on day 7, treatment can be shortened to 12–16 weeks if no dose reduction has been made [13]. For genotype 1 patient with RVR, 24 weeks of treatment is recommended. For patients with cEVR, i.e. HCV RNA undetectable at week 12, 48 weeks of treatment is recommended, whereas 72 weeks of treatment should be considered for patients with partial EVR, i.e. at least a 2 log decrease in HCV RNA levels [8,14,15].

The COBAS TaqMan HCV test is useful for simultaneously analysing qualitative and quantitative HCV RNA or other viral RNA [5,16]. In the near future, we are expecting higher SVR rates in chronic hepatitis C patients treated with new drugs, such as NS3/4A protease inhibitors, NS5A inhibitors and others [4,17], and the COBAS TaqMan HCV test could be useful for selecting patients. The COBAS TaqMan HCV test assay may also be useful for shortening the treatment duration to 16/24 weeks [18]. In the near future, we will use direct antivirals with and later without IFN, HCV RNA testing will be done at weeks 1–4, and HCV RNA negativity will be the preferred parameter for determining the duration of therapy.

In conclusion, our study showed that the COBAS TaqMan HCV test was more sensitive than the heretofore-applied methods, and that it may provide a more accurate prediction of the treatment response, leading to a reduction in adverse events and treatment duration, and to the modification of antiviral regimens in at least some of the patients.

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Senescence marker protein 30 (SMP30)/regucalcin (RGN) expression decreases with aging, acute liver injuries and tumors in zebrafish

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ABSTRACT

Senescence marker protein 30 (SMP30)/regucalcin (RGN) is known to be related to aging, hepatocyte proliferation and tumorigenesis. However, expression and function of non-mammalian SMP30/RGN is poorly understood. We found that zebrafish SMP30/RGN mRNA expression decreases with aging, partial hepatectomy and thioacetamide-induced acute liver injury. SMP30/RGN expression was also greatly decreased in a zebrafish liver cell line. In addition, we induced liver tumors in adult zebrafish by administering diethylnitrosamine. Decreased expression was observed in foci, hepatocellular carcinomas, cholangiocellular carcinomas and mixed tumors as compared to the surrounding area. We thus showed the importance of SMP30/RGN in liver proliferation and tumorigenesis.

1. Introduction

Senescence marker protein 30 (SMP30) was originally identified by two-dimensional gel electrophoresis as a 34 KDa protein of which expression decreases by up to 40% in the aged rat liver without androgen dependence [1]. But it was subsequently found to be the same as regucalcin (RGN), a calcium-related protein without an EF-hand motif [2]. RGN has been intensively studied by Yamaguchi and Takahashi who reported that RGN regulates intracellular Ca^{2+} homeostasis by activating Ca^{2+} pump activity in the cell membrane [3]. Moreover, SMP30/RGN inhibits Ca^{2+}-dependent protein kinase, protein phosphatase and nitric oxidase synthase, and thereby inhibits cell proliferation [4]. SMP30/RGN is known to exist in a broad range of species from mammals to bacteria, and its expression in mammals is high in hepatic parenchymal cells and the renal cortex [5]. Compared with normal mice, SMP30/RGN knockout mice age more rapidly and have increased fat droplets and lysosomes in their livers. Furthermore, SMP30/RGN was shown to be gluconolactonase, an enzyme indispensable for vitamin C synthesis, and the relationship between vitamin C and aging appears to be important [6,7]. SMP30/RGN mRNA expression is reportedly decreased in chemically-induced tumors as compared to surrounding normal liver tissue, based on in situ hybridization [8]. Microarray analysis showed SMP30/RGN to be one of the down-regulated genes in the GST-P positive area [9]. Although overexpression of SMP30/RGN in hepatoma cells down-regulates oncogenes such as c-myc, Haras and c-src [10], details of how SMP30/RGN takes part in the development and progression of cancer remain unknown. Zebrafish are the simplest vertebrate model because of their low breeding costs and the capacity for high-through-put screening [11,12]. Zebrafish provide a good cancer model because various diethylnitrosamine (DEN)-induced tumors resemble human tumors [13]. There are no reports concerning zebrafish SMP30/RGN except a study on dioxin administration to embryos [14]. Moreover, the expression of SMP30/RGN in mixed tumors and cholangiocellular carcinomas has not previously been reported. In this study, we examined the importance of SMP30/RGN in aging, liver proliferation and liver tumorigenesis using the zebrafish model.

2. Materials and methods

2.1. Animals

Zebrafish were maintained in accordance with the Animal care Guidelines of Yamaguchi University. Fish were kept in tap water in plastic tanks and illuminated with fluorescent lighting set at 16 h light and 8 h dark.

2.2. Cell culture

An adult zebrafish liver cell line (ATCC CRL-2643) was cultured in ZFL medium as described by Ghosh et al. [15].

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2.3. DEN exposure

One-year old fish were exposed to 200 ppm DEN for 2 months. DEN solutions were changed every week to compensate for degradation. Following exposure, the fish were maintained in tap water without DEN for 4 months.

2.4. Quantitative RT-PCR

Total RNA was isolated with TRIzol (Invitrogen), and treated with Turbo DNase (Ambion). RT-PCR was performed by utilizing the step one plus real time PCR system and Fast SYBR Green Master Mix (Applied Biosystems). Specific primers for SMP30/RGN (5′-ACT ATG ACA TCC AAA CTG GAG GA-3′ and 5′-CTT CTG TGT CTA TGC ACA TAC CG-3′) were used. Elongation factor 1-alpha was used as an internal control.

2.5. Tissue collection and histology

Fish were killed and opened from the anal vent to the gills. The entire body was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The liver was dissected, dehydrated in alcohol and embedded in paraffin according to routine procedures. Serial sections were cut at a thickness of 3–5 μm. Staining was performed using hematoxylin and eosin (HE).

2.6. Immunohistochemistry (IHC)

Antibody for SMP30/RGN was obtained from Shima Laboratories (Tokyo, Japan). SMP30/RGN was immunohistochemically assessed using the avidin–biotin–peroxidase complex method, as described previously [16].

2.7. Western blot analysis

Samples were prepared by the same methods as previously reported [17]. The blots were incubated for 1 h at room temperature with primary antibodies against SMP30/RGN (Shima), and β-actin (Sigma) in blocking buffer. After being washed, the blots were incubated for 1 h at room temperature with secondary antibodies. Reactive bands were identified using an enhanced chemiluminescence kit (Amersham Biosciences) and autoradiography according to the manufacturer's instructions.

2.8. Partial hepatectomy

Liver regeneration was induced by partial hepatectomy. The ventral lobes of zebrafish livers were removed by the methods reported by Sadler et al. [18].

2.9. Thioacetamide (TAA) treatment

Six-month-old female zebrafish were injected with 300 mg/kg body TAA intraperitoneally. Two days after injection, the livers were collected and used for further examinations.

2.10. Statistical analysis

All data are expressed as means ± S.D. One way ANOVA followed by the Dunnett post hoc multiple comparison test was performed to assess the statistical significance of differences in SMP30/RGN expression with aging. The Kruskal–Wallis test followed by the Steel method was performed to assess the statistical significance of SMP30/RGN expression changes caused by partial hepatectomy. Student's *t*-test was performed to assess the results of other examinations. *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Expression of SMP30/RGN in normal zebrafish tissues

SMP30/RGN is reportedly expressed in the livers and kidneys of mammals. We examined SMP30/RGN expression in various zebrafish tissues by Western blotting methods. The 35 KDa form was observed in livers, intestine and kidneys (Fig. 1A). We then examined immunohistochemical expression patterns and found that SMP30/RGN was expressed in hepatic parenchymal cells and the renal cortex of adult zebrafish (Fig. 1B).

3.2. SMP30/RGN expression changes with aging

Mammalian SMP30/RGN expression is known to decrease with aging. We examined SMP30/RGN expression changes in the liver with aging. We used 3-month-old, 6-month-old, 1-year-old and 3-year-old zebrafish. Body lengths were 19.6 ± 1.9, 22.9 ± 1.9, 28.7 ± 1.5, and 30.3 ± 3.2 mm (Fig. 2A), weights.7 ± 8.3, 295.8 ± 84.7, 493.0 ± 108.3, and 721.3 ± 259.3 mg (Fig. 2B), body mass index (BMI) 0.034 ± 0.002, 0.055 ± 0.009, 0.063 ± 0.008, and 0.076 ± 0.010 g/cm^2 (Fig. 2C), respectively. Three-year old zebrafish had spinal curvature which is a feature of advanced age (Fig. 2D). Fold changes in SMP30/RGN mRNA expression by quantitative RT-PCR were 1.00 ± 0.41,.0 0.43 ± 0.25, 0.41 ± 0.27, and 0.17 ± 0.05, respectively (Fig. 2E). SMP30/RGN expression thus decreased significantly with aging.

3.3. SMP30/RGN expression changes during regeneration after partial hepatectomy

We isolated total RNA from partially hepatectomized livers and regenerating livers after partial hepatectomy, and examined SMP30/RGN mRNA expressions by quantitative PCR with EF1a expression as an internal control. Expression of SMP30/RGN mRNA was significantly decreased to 50.0% ± 13.6% and 50.9% ± 10.4% 1

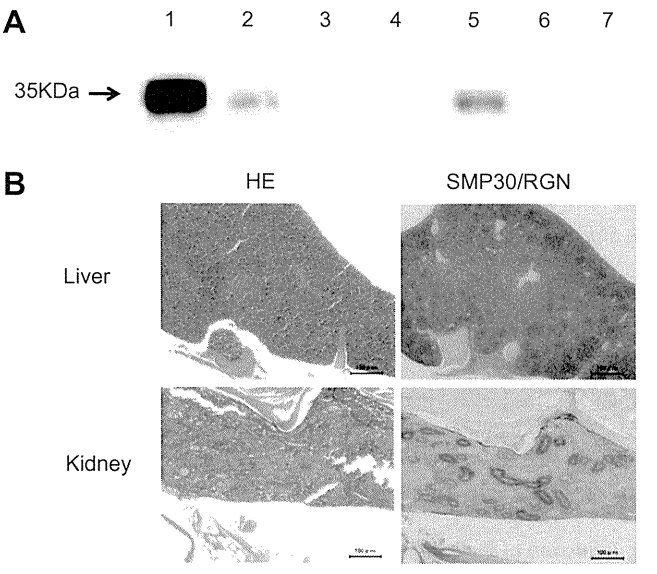

Fig. 1. SMP30/RGN expressions in various tissues in zebrafish. (A) SMP30/RGN expressions in various tissues. 1, liver; 2, intestine; 3, muscle; 4, brain; 5, kidney; 6, heart; 7, testicle. (B) IHC staining of zebrafish liver and kidney with polyclonal antibody for SMP30/RGN. Left: HE staining, right: anti-SMP30/RGN antibody, bar: 100 μm.

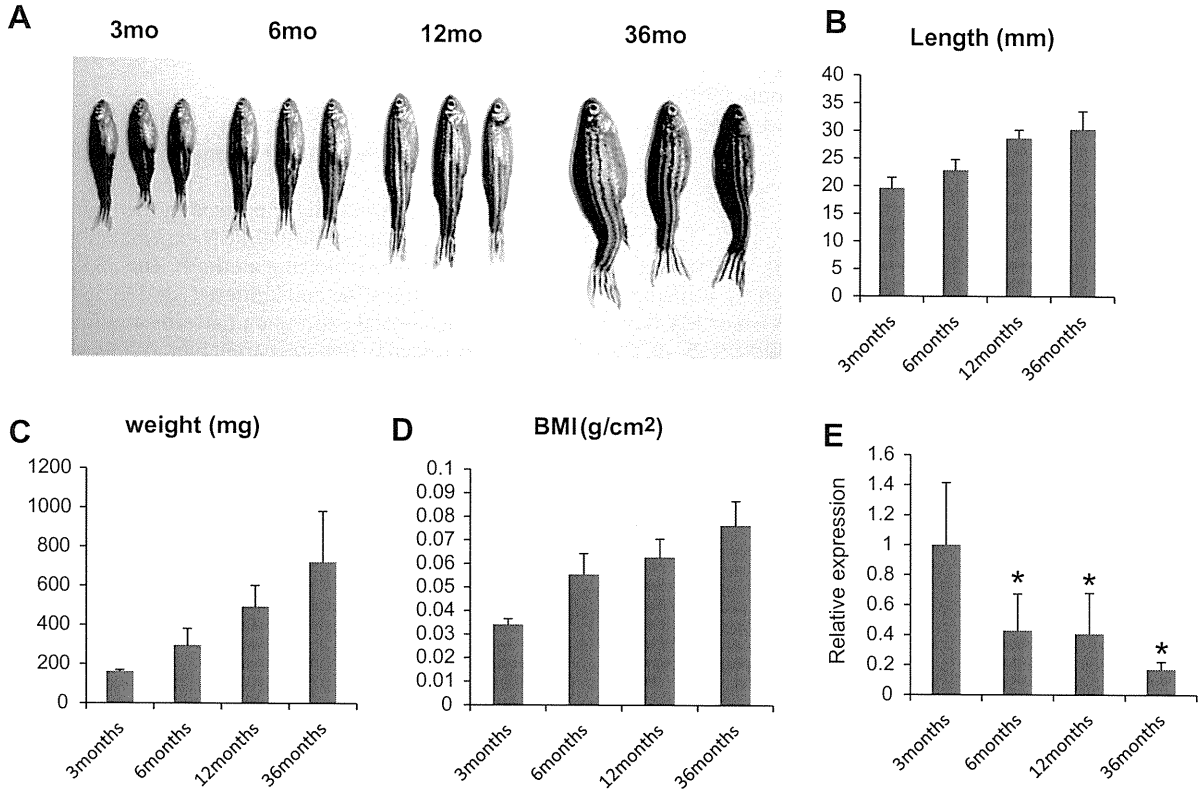

Fig. 2. Change of SMP30/RGN expression with aging. (A) Whole images of zebrafish at 3, 6, 12 and 36 months of age. (B) Body length (cm). (C) body weight (mg). (D) BMI (g/cm^2). Data are expressed as means ± S.D. (E) Expressions of SMP30/RGN mRNA changed with aging, EF1a is used as an internal control. Statistical analysis was performed by one-way ANOVA followed by Dunnett post hoc multiple comparison test, *indicates $P < 0.01$ between 3 months and the groups at 6, 12 and 36 months.

and 2 days after partial hepatectomy, respectively (Fig. 3A). No expression changes were observed 3 and 7 days after partial hepatectomy.

3.4. SMP30/RGN expression changes in acute liver injury induced by TAA administration

We examined expression of SMP30/RGN mRNA in the liver 48 h after intraperitoneal administration of TAA, a hepatotoxic agent, by quantitative RT-PCR. SMP30/RGN mRNA was significantly decreased in TAA-treated livers. Levels of SMP30/RGN expression in control and TAA livers were 1.00 ± 0.08 and 0.58 ± 0.09, respectively (Fig. 3B). The number of PCNA positive cells was increased in TAA-treated liver by IHC (Fig. 3C and D).

3.5. SMP30/RGN expression in zebrafish liver cell line

In order to examine expression of SMP30/RGN in proliferating cells, we used a zebrafish liver cell line (ZFL) (Fig. 4A). ZFL cells were derived from normal adult zebrafish liver, and synthesize and release several proteins into the culture medium, including a 70 kDa protein recognized by anti-bovine serum albumin IgG [15]. SMP30/RGN protein expression was significantly decreased, while PCNA expression was increased in ZFL cells as compared to normal liver extracts (Fig. 4B).

3.6. Induction of liver tumors by DEN administration

We maintained adult zebrafish with 200 ppm DEN solution for 2 months with weekly solution changes, and then kept them in tap water without DEN for 4 months to allow liver tumor development. We obtained some zebrafish with bulging abdomens (Fig. 4C). We then dissected these zebrafish and confirmed liver enlargement (Fig. 4D and D′).

3.7. SMP30/RGN expression in zebrafish liver tumors

We examined SMP30/RGN expressions in various liver tumors by IHC. Decreased SMP30/RGN expression and increased PCNA expression were observed in foci as compared to surrounding normal liver tissues (Fig. 4F and G). Decreased expression was observed in hepatocellular carcinomas (HCC) (Fig. 4I), which are characterized by complete loss of normal tubular architecture and compression of the peripheral area on HE staining (Fig. 4H). Increased PCNA-positive staining in the nuclear was also seen in HCC (Fig. 4J). We noted lower expressions in cholangiocellular carcinomas, which have highly developed ductal structures (Fig. 4K–M). We also recognized decreased SMP30/RGN expression in mixed cell tumors, which have irregular cords of small hepatocytes and small ductules of biliary epithelial cells (Fig. 4N–P). We checked all 18 DEN-treated zebrafish, and found three to have foci, six adenoma/HCC, three cholangiocellular carcinomas and four for mixed type tumors. We examined SMP30/RGN expression in each tumor by IHC, and found decreases in three of the four foci (75%), 15 of the 17 adenoma/HCC (88.2%), all three cholangiocellular carcinomas (100%) and all six mixed tumors (100%) (Table 1).

4. Discussion

SMP30 was originally reported to be a protein which is greatly reducing with aging [1]. Although the existence of SMP30/RGN was observed in various organisms, there are few reports concerning non-mammalian SMP30/RGN. Detailed SMP30 examination was reported for abalone, a marine invertebrate [19], but detailed

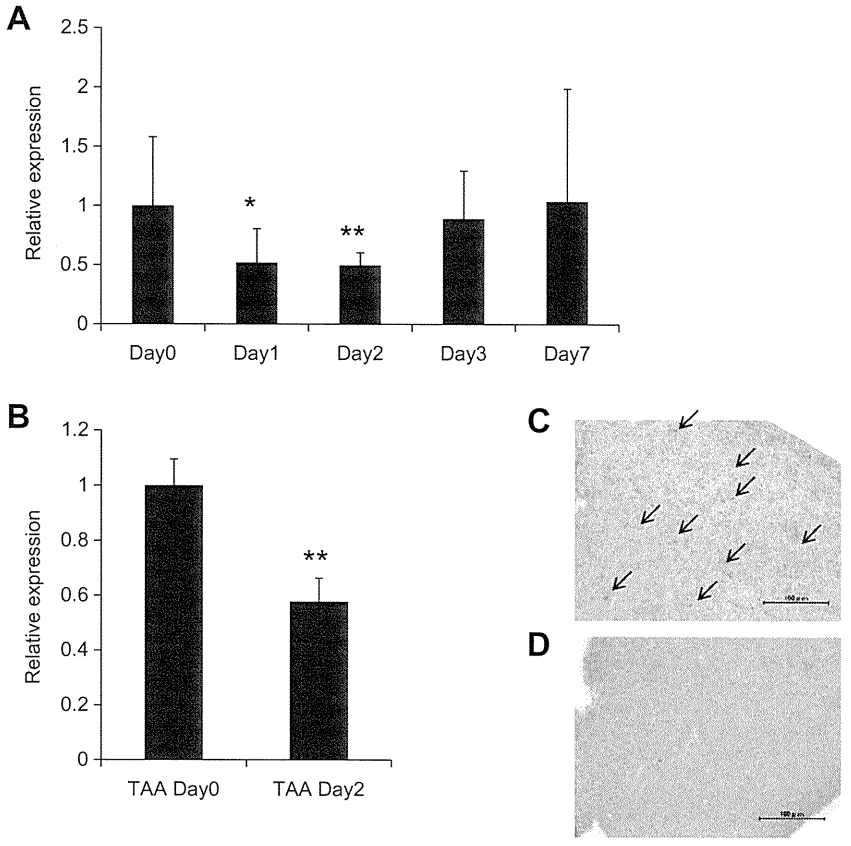

Fig. 3. SMP30/RGN mRNA expression in liver regeneration after partial hepatectomy and liver injury after TAA injection. (A) Expressions of SMP30/RGN mRNA after partial hepatectomy * and ** indicate $P < 0.05$ and 0.01 between control (Day 0) and partial hepatectomy group (Days 1–3 and 7). (B) SMP30/RGN mRNA expression changed in TAA-treated livers. (C) PCNA expression in TAA-treated liver, bar: 100 μm. Arrow indicated PCNA-positive cells. (D) PCNA expression incontrol liver. Bar: 100 μm.

examinations of zebrafish SMP30/RGN are lacking. We examined SMP30/RGN in zebrafish which is a good model for tumorigenesis and high-through-put screening is possible.

Zebrafish are becoming a new aging model [20]. Being different from mammals, teleosts keep growing in adulthood. In this study, 3-year-old zebrafish had 1.6 times the body length of a 6-month zebrafish. We examined whether SMP30/RGN mRNA expression decreases in the growing liver. We found SMP30/RGN mRNA expression to be significantly decreased with aging (Fig. 2E). SMP30/RGN has antioxidant properties that protect cells from oxidative stress, and overexpression of SMP30/RGN in a liver cell line contributed to a marked decrease in reactive oxygen species (ROS) formation as well as decreased lipid peroxidation, superoxide dismutase activity and glutathione levels [21]. Down-regulation of SMP30/RGN during the aging process may increase ROS formation. Moreover, calcium metabolism is important for understanding the aging process because it is related to electrolyte balance, calculus formation and osteoporosis. From the viewpoint of calcium metabolism, calbindin-D, a calcium binding protein, shows similar age-associated decreases in the brains of rats and humans. There is a possibility that the age-associated decrease in SMP30/RGN mRNA expression is related to calcium homeostasis, which deteriorates with aging.

SMP30/RGN mRNA expression is reportedly decreased 12 h after 70% partial hepatectomy, and then increased 2–3 days thereafter [22]. Zebrafish SMP30/RGN mRNA expression is decreased 1–2 days after partial hepatectomy. Overexpression of SMP30/RGN in hepatoma cells suppresses the expression of c-src but induces p53 and Rb expressions [23]. It is reasonable that SMP30/RGN expression is suppressed within 2 days after partial hepatectomy, when hepatocytic proliferation activity is high. However, in contrast to the mouse SMP30/RGN expression data, we found no increase in expression after partial hepatectomy. Partial hepatectomy is achieved in zebrafish by removing about 30% of the total liver, and the PCNA index peaks 48 h thereafter [18]. On the other hand, mouse partial hepatectomy requires that more than 70% of the liver be removed. There is a possibility that the zebrafish SMP30/RGN expression pattern differs from that in mice due to the smaller amount of liver removed. On the other hand, there was reportedly no increase in expression after partial hepatectomy in the mouse. A microarray analysis revealed that SMP30/RGN expression was decreased 12 h after partial hepatectomy, but there were no expression changes 1–2 days after partial hepatectomy [24]. The difference in expressions and the role of SMP30/RGN after partial hepatectomy require further investigation.

TAA is a centrilobular hepatotoxic agent which is metabolized by CYP2E1. Mice receiving a single dose of TAA are used as an acute liver injury model [25]. In this study, SMP30/RGN mRNA expression was decreased 48 h after TAA administration. TAA administration caused centrilobular necrosis and there was a subsequent regenerative response. As after partial hepatectomy, SMP30/RGN expression diminished when the liver was in a proliferative state.

Changes in SMP30/RGN expression in some tumor types such as breast cancer, prostate cancer and HCC were reported [26]. At the transcriptional level, SMP30/RGN mRNA was profiled as being a down-regulated gene in rat GST-P positive DEN-induced lesions as compared to the surrounding area [9]. A clinical proteomic study showed SMP30/RGN expression to be decreased in tumors [27].

In this study, we found expression of SMP30/RGN protein to be decreased in a zebrafish liver cell line (Fig. 4B) and in DEN-induced

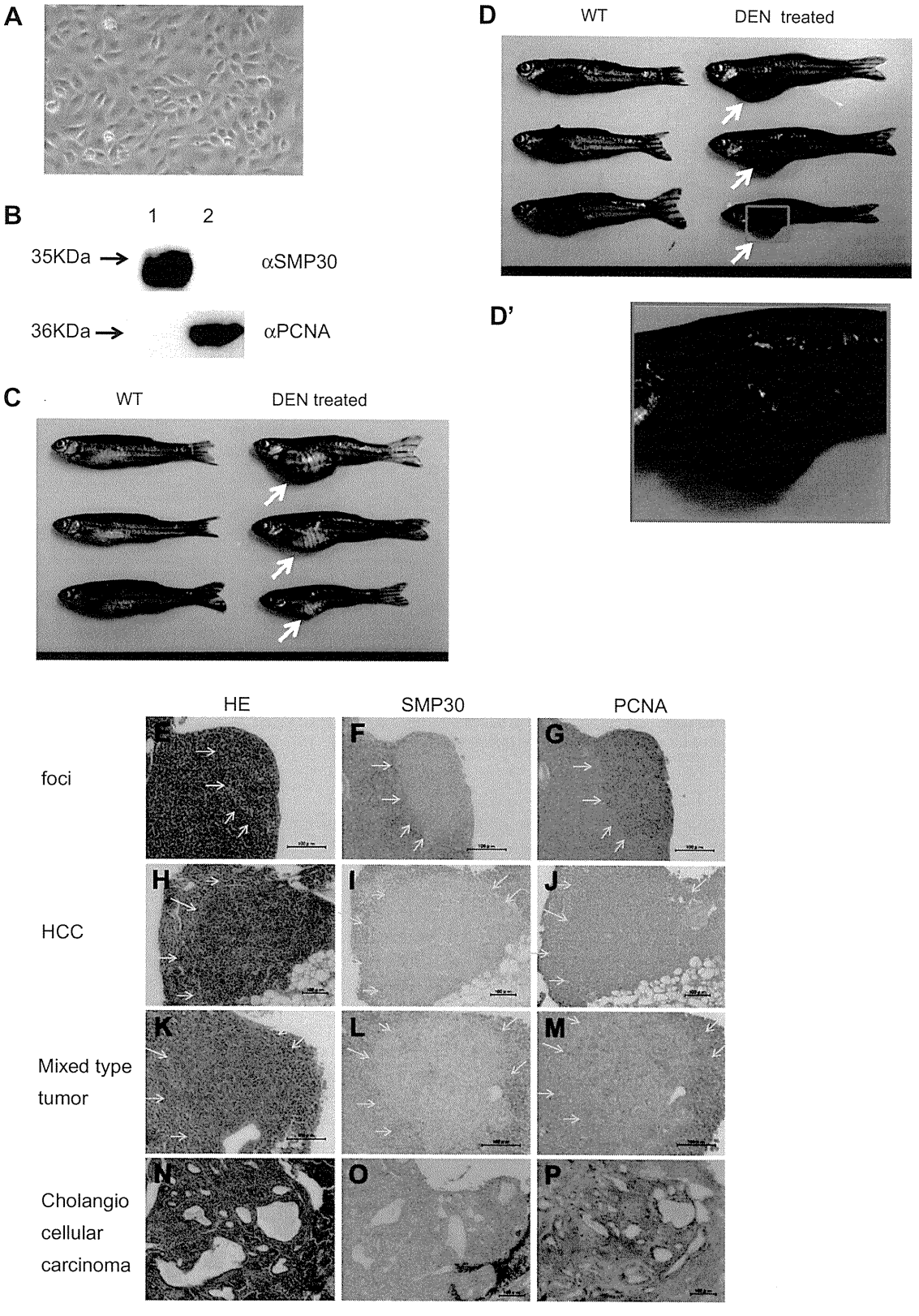

Fig. 4. SMP30/RGN expression in zebrafish liver tumor. (A) Photomicrograph of ZFL. (B) Expressions of SMP30/RGN and PCNA in normal liver and zebrafish liver cell line. (C) Whole image of DEN-treated zebrafish: arrows indicate bulging abdomen. (D) Image from DEN-treated zebrafish: arrows indicate liver tumor. (D′) High-power field of swollen liver. (E–P) IHC analyses of various liver tumors. Left: HE staining, middle: anti-SMP30/RGN antibody, right: anti-PCNA antibody, bar: 100 μm. (E–G) foci, (H–J) HCC, (K–M) mixed type tumor, (N–P) cholangiocellular carcinoma. Arrows indicate boundary of tumor and surrounding area.

foci and adenoma/HCC, as compared to surrounding tissues (Fig. 4E–J). Furthermore, SMP30/RGN expression was decreased in mixed tumors and cholangiocellular carcinomas. This is, to our knowledge, the first report of SMP30/RGN expressions in these

Table 1
Expression of SMP30/RGN in zebrafish tumors induced by DEN treatment.

	Number of fish with tumors/ total number of fish examined	Total number of tumors	Number of tumors with decreased SMP30 expression	Ratio of SMP30 decreased tumors (%)
Foci	3/18	4	3	75
Adenoma/HCC	6/18	17	15	88
Mixed tumor	4/18	6	6	100
Cholangiocellular carcinoma	3/18	3	3	100

two tumors. Mixed tumors are a rare type of primary hepatic tumor thought to originate from a single site, but with two possible endpoints of differentiation. Cholangiocellular carcinoma is an adenocarcinoma and the actual cell origin is unknown, although it has been suggested to arise from pluripotent hepatic stem cells. Overexpression of SMP30/RGN in HepG2 suppresses cell proliferation and expressions of oncogenes, while increasing the expressions of tumor suppressor genes [28]. In addition, the frequency of antibody against SMP30/RGN is significantly higher in well-differentiated than in poorly-differentiated HCC [26]. We thus speculate that reduced SMP30/RGN expression enhances cell proliferation and is ultimately related to tumor progression. SMP30/RGN expression reduction was observed at a high frequency in tumors (Table 1). SMP30/RGN would thus be a good marker for liver various tumors.

In conclusion, we demonstrated the importance of SMP30/RGN in aging, partial hepatectomy, acute liver injury and liver tumorigenesis. SMP30/RGN has multiple functions in aging, proliferation and tumorigenesis, but physiological roles of SMP30/RGN have yet to be fully elucidated. Detailed investigations of the functions of SMP30/RGN are needed.

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Molecular Gastrointestinal Medicine

特集 消化器癌のCancer Stem Cell, Cancer Initiating Cell

肝癌幹細胞を探る

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KEY WORDS

肝癌幹細胞，肝前駆細胞，肝細胞癌

SUMMARY

肝細胞癌は肝硬変の進展に伴い発癌率が増加する．つまりは線維化進行というニッチは，発癌が誘導されやすい環境であり，肝細胞癌は多中心性に発癌する．近年のfluorescence activated cell sorting（FACS）技術の進歩による肝臓構成細胞の分離技術の進歩，また人工多能性幹細胞（induced pluripotent stem cell：iPS細胞）の発見より“成熟した細胞も簡単に初期化する”ことが明らかになり，従来とは違う研究アプローチ，視点から，肝細胞癌のなかの抗癌剤耐性の癌幹細胞（cancer stem cell）の研究が進められ，新たに“肝癌幹細胞”の報告があいついできた．また肝癌幹細胞の制御機構についても，メチル化，マイクロRNA（miRNA）による制御，transforming growth factor（TGF）-βなど各種サイトカインとの関連などさまざまな報告がされてきた．本稿においては，最近の肝癌幹細胞の研究について紹介し，従来の肝発癌の考えと比較し，その重要について概説する．

はじめに

肝細胞癌の発生率は，日本国内では全癌のなかで第4位である．肝線維化の進行，すなわち慢性肝炎から肝硬変症の進行とともに，肝発癌率が増加する．肝障害の進行に伴い，肝線維化が進行し，その結果多中心性の発癌を示す．肝硬変進行のなかでの肝前駆細胞の発生，増殖の理解が重要である．肝臓領域において最近になり急速に肝幹細胞，肝癌幹細胞の研究成果が集積している．この一因としては，モノクローナル抗体およびfluorescence activated cell sorting（FACS）技術にて，肝臓構成細胞を細胞膜抗原の発現により厳密に識別・分離して性状を解析することできるようになってきたことによる．肝癌幹細胞の発見は，癌自体はヘテロな細胞集団であり，そのなかに抗癌剤耐性の癌幹細胞（cancer stem cell）があることが明らかになりつつあり，癌の制御の問題を考えるうえで重要である．一方で，従来の肝発癌の考え方との協調的な理解は十分でない．この理由としては，従来の肝発癌の理解では，dysplastic noduleなどの前癌性病変を経て肝細胞癌ができるという考えであった

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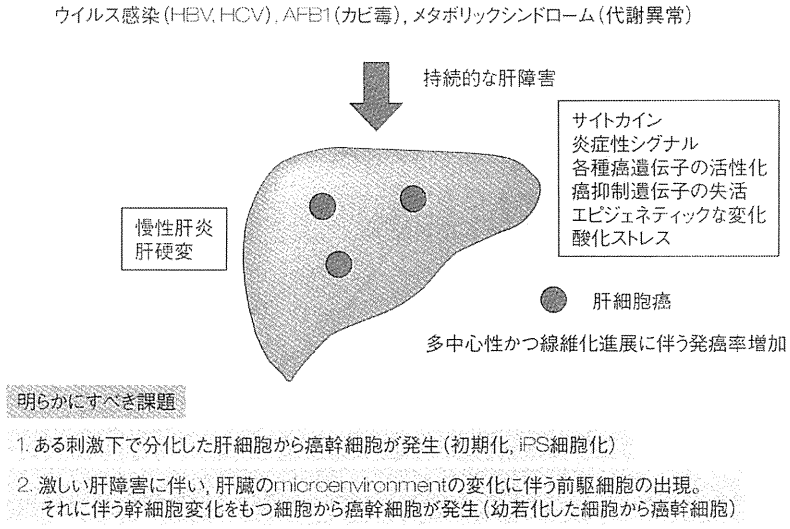


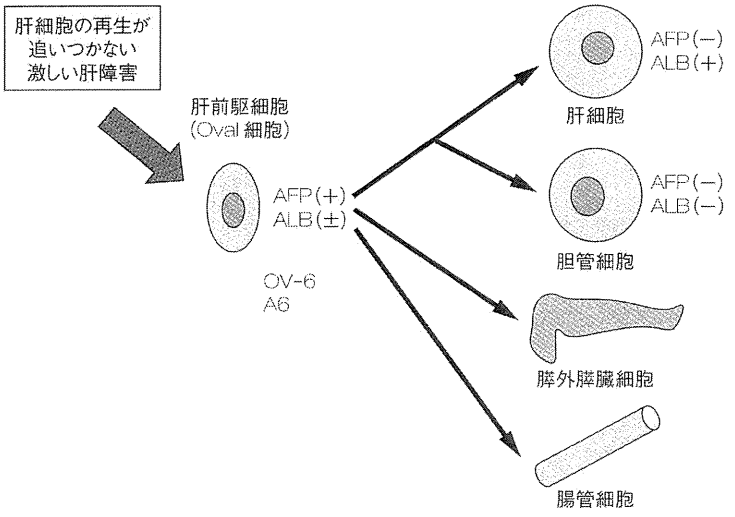
図❶　肝細胞癌の特徴と癌幹細胞の意味

が，人工多能性幹細胞（induced pluripotent stem cell：iPS細胞）の発見などより，成熟した細胞も特殊な刺激下では簡単に初期化することが明らかになったことより，成熟肝細胞から癌幹細胞が発生する可能性は十二分に推察されることになった．図❶では簡単に，今の肝癌の知見，および本稿でより明らかにしたい点を提示する．それにより肝癌幹細胞の新たな知見を理解してもらい，今後の肝癌幹細胞研究の重要性について論じたい．

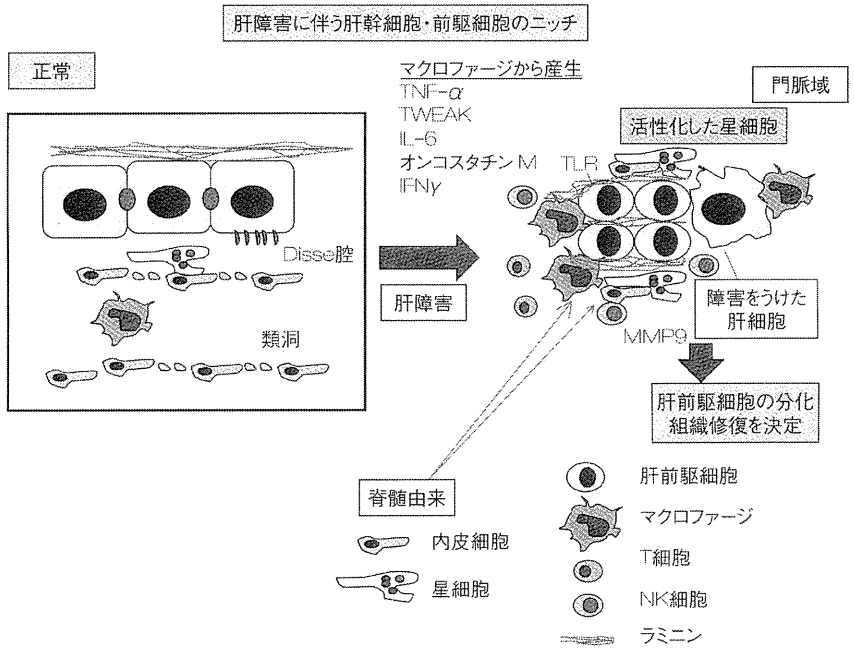
肝幹細胞，前駆細胞と肝癌幹細胞

“幹細胞”の定義としては，“自己複製能と分化能をもった細胞”で，一番明確なのが造血幹細胞である．骨髄中の造血幹細胞は生涯にわたり維持され，大量の血球をつくりつづける．細胞表面マーカーを使って純化した造血幹細胞を一個移植すれば，それに由来する血球が何ヵ月もつくられるので，造血幹細胞についてはきわめて厳密に定義される．肝幹細胞は幹細胞の厳密な定義にはあてはまらないものの，“クローン増殖ができて，しかも胆管と肝細胞への分化能をもった細胞”が“肝幹細胞”と考えられている．一方，前駆細胞の増殖は一過性であり，長期に増殖しない細胞である．発生過程で出現する肝芽細胞は，増殖能と2方向への分化能をもった肝幹細胞とみなされる[1,2]．また，成体肝臓の重篤な肝障害に伴い，肝細胞，胆管細胞の両方の性質をもった細胞が門脈周辺に発生するが，この細胞の核が卵円形のであることから，Oval細胞ともよばれている．Oval細胞は胆管細胞，肝細胞に分化することから（図❷），従来，肝幹細胞とみなされていた．しかし，最近の研究では，大部分のOval細胞は一過性に増殖する前駆細胞であると考えられている．Oval細胞の起源は胆管と肝細胞索のあいだのヘリング管との説が有力であったが，細胞系譜解析から肝細胞からも発生するとの報告もあり，その起源については議論があり明らかでない．図❸は，肝幹細胞，肝前駆細胞の制御機構についての最近の知見をまとめたものであるが，マクロファージ，肝星細胞などから構成されるさまざまな分化ニッチの制御により，肝幹細胞は制御される．またこのような前駆細胞の発生をつうじてまた逆に周囲の環境を制御し，その結果組織修復が進むと考えられる[3]．

癌幹細胞の概念そのものは150年前の“embryonal rest hypothesis”を基盤としており，“癌細胞は胎児期の細胞と同じ表現型”をとることから提唱された．多くの癌組織は階層性をもった不均一な細胞集団であり，癌組織中の一部の細胞のみが増殖能と分化能をもち，それらは免疫不全マウスに移植すると癌をつくることができることから，“tumor initiating cell”ともよばれている．こうした癌幹細胞の存在は，白血病[4]，脳腫瘍[4]，乳癌[5]をは

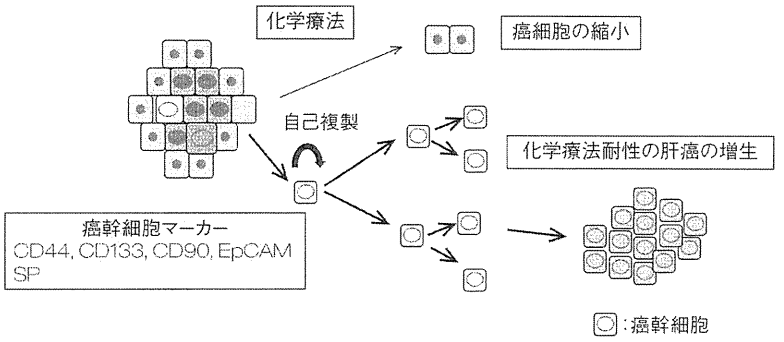


図❷ 肝前駆細胞（Oval 細胞）の分化〔AAF（Acetylaminofluorene）/PH（partial hepatectomy：部分肝切除）モデルより〕
AAF を投与し肝再生ができない．ALB：アルブミン



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



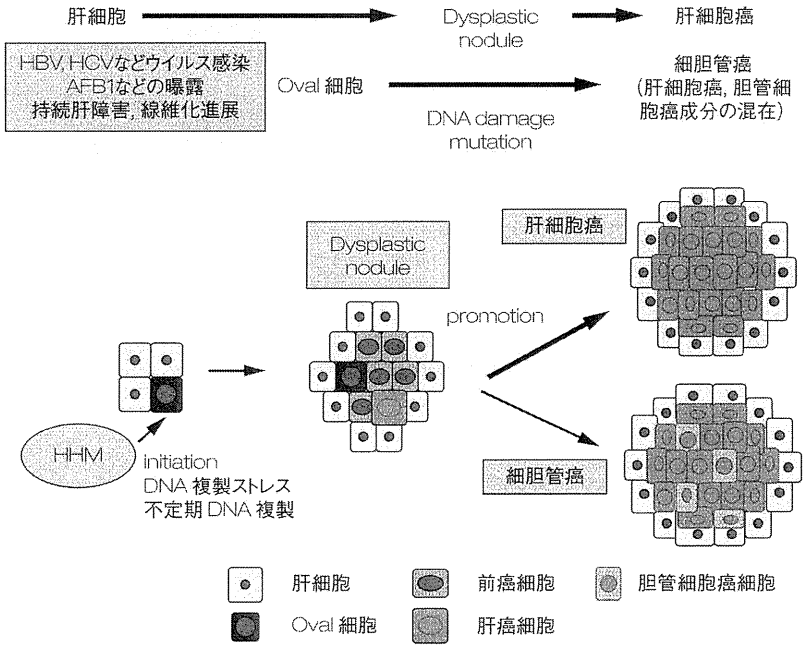
図❹　抗癌剤耐性から考えた癌幹細胞

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



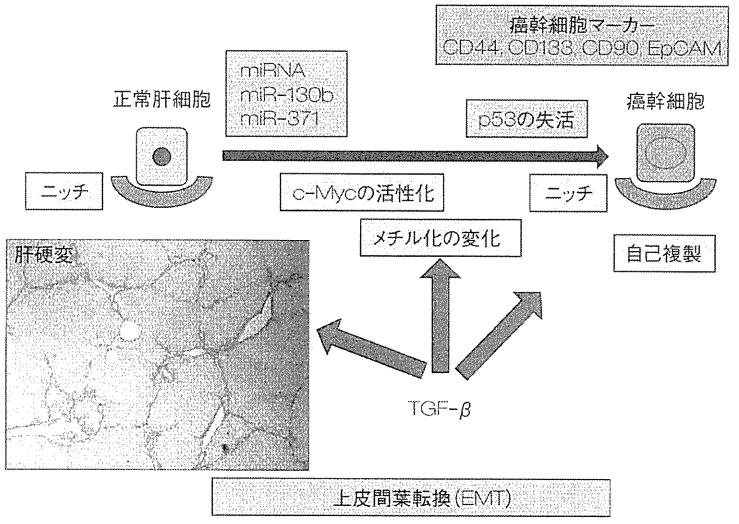
図❺ 肝発癌（肝細胞癌の発生，細胆管癌の発生）

（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



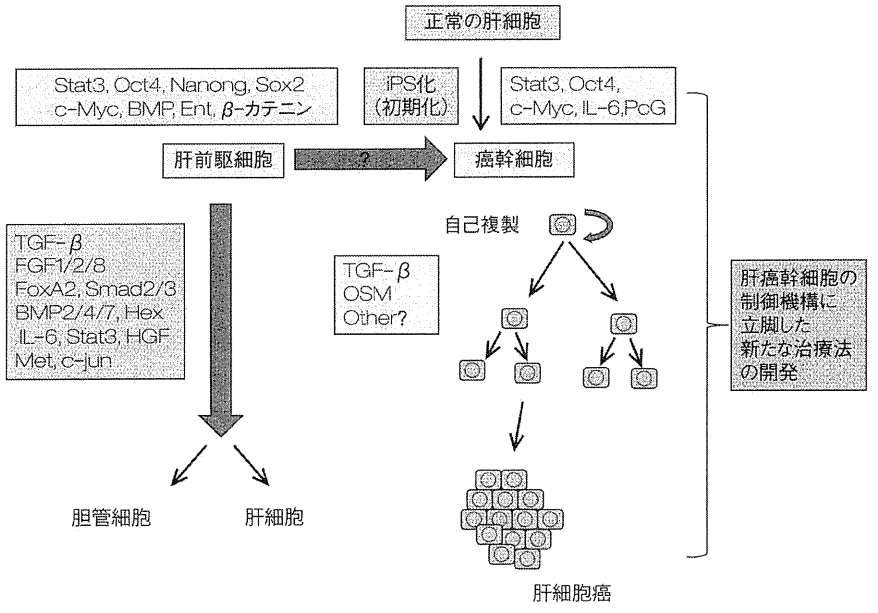
図❻　癌幹細胞の発生

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．

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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 ± 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