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# SEK1/MKK4-Mediated SAPK/JNK Signaling Participates in Embryonic Hepatoblast Proliferation via a Pathway Different from NF-κB-Induced Anti-Apoptosis

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**Mice lacking the stress-signaling kinase SEK1 die from embryonic day 10.5 (E10.5) to E12.5. Although a defect in liver formation is accompanied with the embryonic lethality of *sek1*$^{-/-}$ mice, the mechanism of the liver defect has remained unknown. In the present study, we first produced a monoclonal antibody specifically recognizing murine hepatoblasts for the analysis of liver development and further investigated genetic interaction of *sek1* with tumor necrosis factor-α receptor 1 gene (*tnfr1*) and protooncogene *c-jun*, which are also responsible for liver formation and cell apoptosis. The defective liver formation in *sek1*$^{-/-}$ embryos was not protected by additional *tnfr1* mutation, which rescues the embryonic lethality of mice lacking NF-κB signaling components. There was a progressive increase in the hepatoblast cell numbers of wild-type embryos from E10.5 to E12.5. Instead, impaired hepatoblast proliferation was observed in *sek1*$^{-/-}$ livers from E10.5, though fetal liver-specific gene expression was normal. The impaired phenotype in *sek1*$^{-/-}$ livers was more severe than in *c-jun*$^{-/-}$ embryos, and *sek1*$^{-/-}$ *c-jun*$^{-/-}$ embryos died more rapidly before E8.5. The hepatoblast proliferation required no hematopoiesis, since liver development was not impaired in *AML1*$^{-/-}$ mice that lack hematopoietic functions. Stimulation of stress-activated protein kinase/c-Jun N-terminal kinase by hepatocyte growth factor was attenuated in *sek1*$^{-/-}$ livers. Thus, SEK1 appears to play a crucial role in hepatoblast proliferation and survival in a manner apparently different from NF-κB or c-Jun.** 

***Key words:*** **SEK1; NF-κB; c-Jun; HGF; hematopoiesis; hepatogenesis.**

## INTRODUCTION

Embryonic liver formation consists of multiple stages and is influenced by hormonal factors as well as intercellular and matrix–cellular interactions. In mice, the initial event of liver ontogeny occurs around embryonic day 9 (E9), when epithelial cells of the foregut endoderm commit to becoming the liver primordium through their interaction with the cardiogenic mesoderm. The liver primordium proliferates and invades the mesenchyme of the septum transversum to give rise to the hepatic codes and buds at

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E9.5. A critically genetic checkpoint in embryogenesis is the switch from yolk sac- and aorta–gonad–mesonephros region-dependent blood formation to liver-dependent hematopoiesis. This switch in hematopoietic organs occurs from E10.5 to E12.5. The next major stage occurs around E14.5, when both hepatocytes and bile-duct epithelial cells arise embryologically from a common founder cell, the hepatoblast, which has bipotential differentiation capabilities in liver development (LeDouarin, 1975; Houssaint, 1980).

The degree of hepatic maturation has been characterized by the expression of liver- and stage-specific genes (Derman *et al.,* 1981; Panduro *et al.,* 1987). Alphafetoprotein (AFP) is an early fetal hepatic marker (E9), and its expression decreases as the liver develops (Shiojiri *et al.,* 1991). In contrast, expression of albumin, the most abundant protein synthesized by hepatocytes, starts in early fetal hepatocytes (E12) and reaches the maximal level in adults (Tilghman and Belayew, 1982). However, antibodies specific for AFP or albumin are not adequate to estimate the precise numbers of hepatoblasts in the early fetal livers at E9.5–E12.5 because both are diffusible serum proteins. Therefore, novel antibodies clearly and specifically recognizing individual hepatoblasts are required for studying fetal liver development in detail.

Recently, it has been shown that close proximity of cardiac mesoderm, which expresses fibroblast growth factors (FGFs) 1, 2, and 8, causes the foregut endoderm to develop into the liver by using ventral foregut endoderm isolated from mouse embryos at E8.25 (Jung *et al.,* 1999; Zaret, 2000). On the other hand, another recent report has indicated that a paracrine mechanism of the late hepatogenesis is derived from E14.5 murine embryos in cultured fetal liver cells. Blood cells in the fetal liver produce an interleukin (IL) 6 family cytokine, oncostatin M (OSM), to promote the development of hepatocytes (Kamiya *et al.,* 1999). However, the relationship between hepatogenesis and hematopoiesis in early fetal liver development remains unclear.

Stress-activated protein kinase/extracellular signal-regulated kinase kinases, SEK1/MKK4 and SEK2/MKK7, are direct activators of the stress-activated protein kinase SAPK (also called c-Jun N-terminal kinase, JNK). Both are activated in response to a variety of cellular stresses, such as changes in osmolarity, metabolic poisons, DNA damage, heat shock, or inflammatory cytokines, IL-1 and tumor necrosis factor-$\alpha$ (TNF-$\alpha$). SEK1 and/or MKK7-mediated activation of SAPK/JNK phosphorylates c-Jun and activates c-Jun/Fos heterodimeric AP-1 transcriptional complexes (Davis, 2000; Chang and Karin, 2001). Several groups, including us, have disrupted the *sek1* gene in mice by using homologous recombination (Nishina *et al.,* 1997a,b, 1999; Yang *et al.,* 1997; Ganiatsas *et al.,* 1998). SEK1-deficient embryos displayed severe anemia and died between E10.5 and E12.5. Hematopoiesis from yolk sac precursors and vasculogenesis are normal in SEK1-deficient embryos. However, hepatogenesis and liver formation were severely impaired in the mutant embryos, and SEK1-deficient embryos had a greatly reduced number of hepatocytes at E11.5–E12.5. Whereas formation of the primordial liver and hepatic bud appeared to be normal, SEK1-deficient hepatocytes underwent massive apoptosis at E12.5 (Ganiatsas *et al.,* 1998; Nishina *et al.,* 1999). Embryos lacking the *c-jun* gene also display defective liver organization and die between E11.5 and E15.5 (Hilberg *et al.,* 1993; Johnson *et al.,* 1993). These results indicate that SEK1 and c-Jun provide a crucial and specific survival signal for fetal hepatogenesis. It is as yet unclear whether SEK1 plays a role in hematopoietic cells or in hepatogenesis, what receptors trigger SEK1 activation, and what molecules regulate the SEK1-mediated signaling pathway in fetal liver development.

TNF$\alpha$ elicits a wide range of biological responses, such as inflammation, tumor necrosis, differentiation, cell proliferation, and apoptosis, through the stimulation of its receptor, TNFR1. Recently, it has been revealed that three separated signaling pathways, the induction of apoptosis, NF-$\kappa$B activation, and SEK1- and/or MKK7-mediated SAPK/JNK activation, are simultaneously mediated through TNFR1 and that SAPK/JNK activation appears not to be involved in TNFR1-dependent induction of apoptosis, while activation of NF-$\kappa$B protects against the apoptosis (Liu *et al.* 1996). Knockout mice of genes that are involved in NF-$\kappa$B signaling result in massive liver degeneration and apoptosis during midgestation at E12.5–E16. These include the RelA subunit of transcription factor NF-$\kappa$B (died at E15–E16; Beg *et al.,* 1995), I$\kappa$B kinase $\beta$/IKK2 (died at E12.5–E14; Q. Li *et al.,* 1999; Z. W. Li *et al.,* 1999; Tanaka *et al.,* 1999), NEMO/IKK$\gamma$ (died at E12.5–E13.0; Rudolph *et al.,* 2000), and TRAF2-associated kinase, T2K (died at E12.5–E14.5; Bonnard *et al.,* 2000). Importantly, the embryonic lethality and liver apoptosis observed in RelA-, IKK2-, or T2K-knockout mice can be rescued by the simultaneous inactivation of TNFR1, suggesting that the apoptosis is induced by TNF-$\alpha$ circulating in the embryos (Q. Li *et al.,* 1999; Bonnard *et al.,* 2000; Rosenfeld *et al.,* 2000). However, a physiological role of the SEK1 and/or MKK7-mediated activation of SAPK/JNK in response to TNFR1 ligation remains to be resolved (Liu *et al.,* 1996).

To understand the mechanisms of defective liver formation, we prepared monoclonal antibodies specifically recognizing murine fetal livers and characterized them using paraffin sections at various stages of embryos. One of them, called anti-Liv2, recognized specifically so-called hepatoblasts at E9.5–E12.5. We examined the relationship between the lethality of $sek1^{-/-}$ embryos and TNFR1-mediated apoptosis in mice lacking these genes. We found that $sek1^{-/-}$ $tnfr1^{-/-}$ double mutant embryos delay the beginning of liver resorption compared with $sek1^{-/-}$ $tnfr1^{+/-}$ embryos by 2 days and that the liver defect is not rescued by $sek1^{-/-}$ $tnfr1^{-/-}$ genotype. In addition, we investigated the ability of hepatoblast growth, as judged by incorporation of bromo deoxyuridine (BrdU), in $sek1^{-/-}$ embryos at E10.5 before the occurrence of defective liver formation and massive apoptosis. We found a growth defect of hepatoblasts in $sek1^{-/-}$ embryos at E10.5. Furthermore, we showed that hepatoblasts could develop without hematopoiesis in

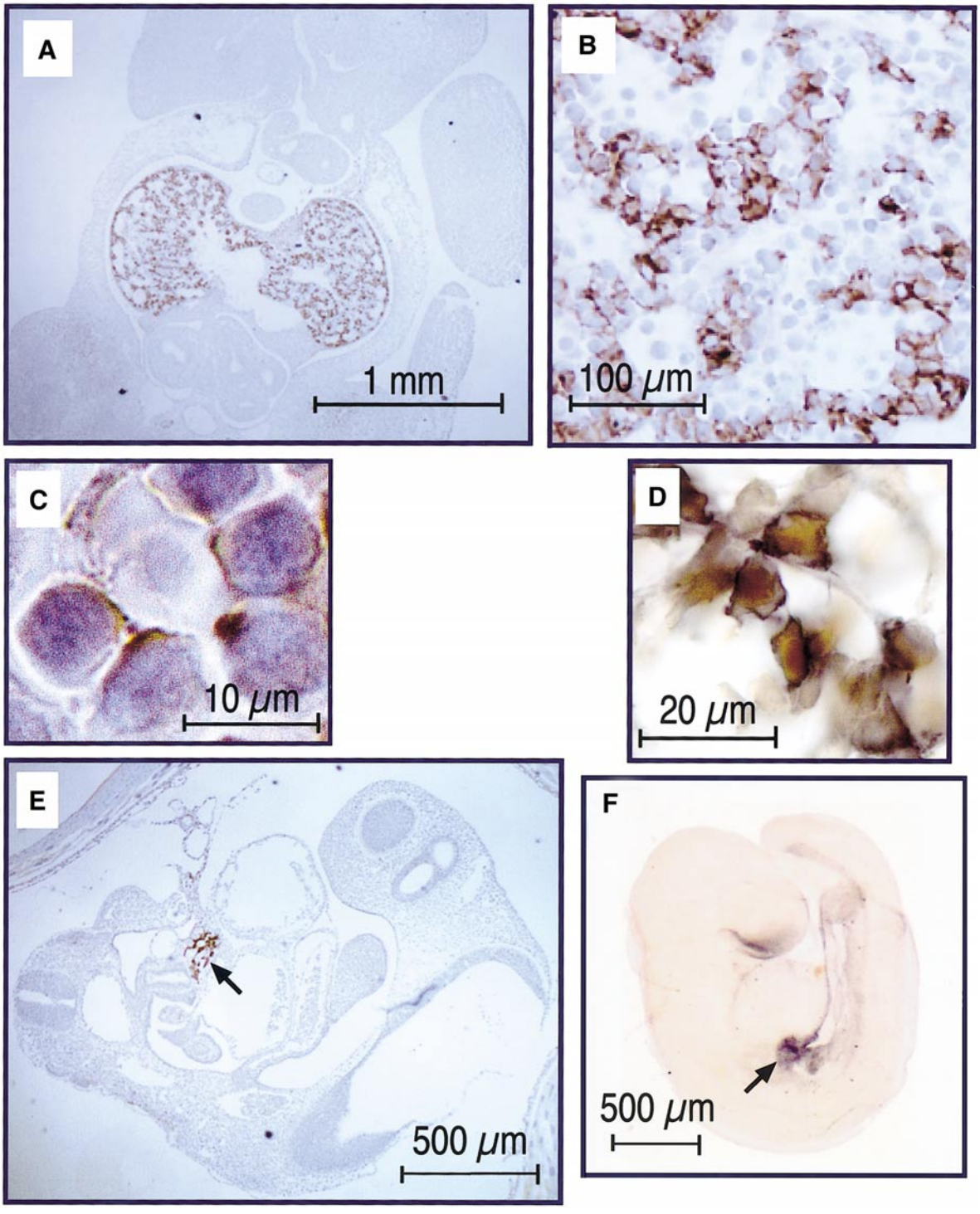


**FIG. 1.** Characterization of a novel rat monoclonal antibody, anti-Liv2; the specific recognition of hepatoblasts in murine embryos. Transverse paraffin-sections of murine embryonic livers at E11.5 (A–) and E9.5 (E), together with a frozen section at E11.5 (D) and a whole embryo at E9.5 (F), were stained with a rat monoclonal antibody, anti-Liv2. Positive cells exhibit a brown (A–C, E) or dark blue (D, E) precipitate. (D) The frozen section containing Liv2-positive cells (dark blue) was further stained with anti-HNF-3$\beta$ (brown). The arrows show hepatic buds.

**TABLE 1**

Analysis of Embryos Obtained from $sek1^{+/-}$ $tnfr1^{-/-}$ Intercrosses or $sek1^{+/-}$ $tnfr1^{+/-}$ and $sek1^{+/-}$ $tnfr1^{+/+}$ Intercrosses

| Embryonic stage | $sek1^{+/+}$ $tnfr1^{+/-}$ | $sek1^{+/-}$ $tnfr1^{+/-}$ | $sek1^{-/-}$ $tnfr1^{+/-}$ | $sek1^{+/+}$ $tnfr1^{-/-}$ | $sek1^{+/-}$ $tnfr1^{-/-}$ | $sek1^{-/-}$ $tnfr1^{-/-}$ |
|---|---|---|---|---|---|---|
| E12.5 | 4 | 6 | 1* | 12 | 34 | 15 |
| E13.5 | 11 | 23 | 6* | 17 | 33 | 17 |
| E14.5 | n.d. | n.d. | n.d. | 9 | 16 | 1* |

*Note.* Embryos were isolated at the indicated time points of gestation and analyzed for Mendelian ratios of all three expected genotypes. Genotypes of embryos were determined by PCR, and the numbers of each genotype are listed below. Asterisks (*) indicate that embryos were under the conditions of resorption. n.d., not determined.

early fetal liver at E9.5–E11.5 using $AML1^{-/-}$ embryos that lack definitive hematopoiesis (Okuda *et al.*, 1996; Wang *et al.*, 1996). Activation of SAPK/JNK by hepatocyte growth factor (HGF) observable in wild-type fetal livers was markedly impaired in $sek1^{-/-}$ fetal livers. Thus, the lethality of *sek1* mutant embryos is most likely due to a defect reflecting SEK1 function not associating with NF-κB-induced anti-apoptosis in the cell growth of hepatoblasts.

## MATERIALS AND METHODS

### *Mice*

C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). SEK1, c-Jun, AML1, and TNFR1 mutant mice were gener-

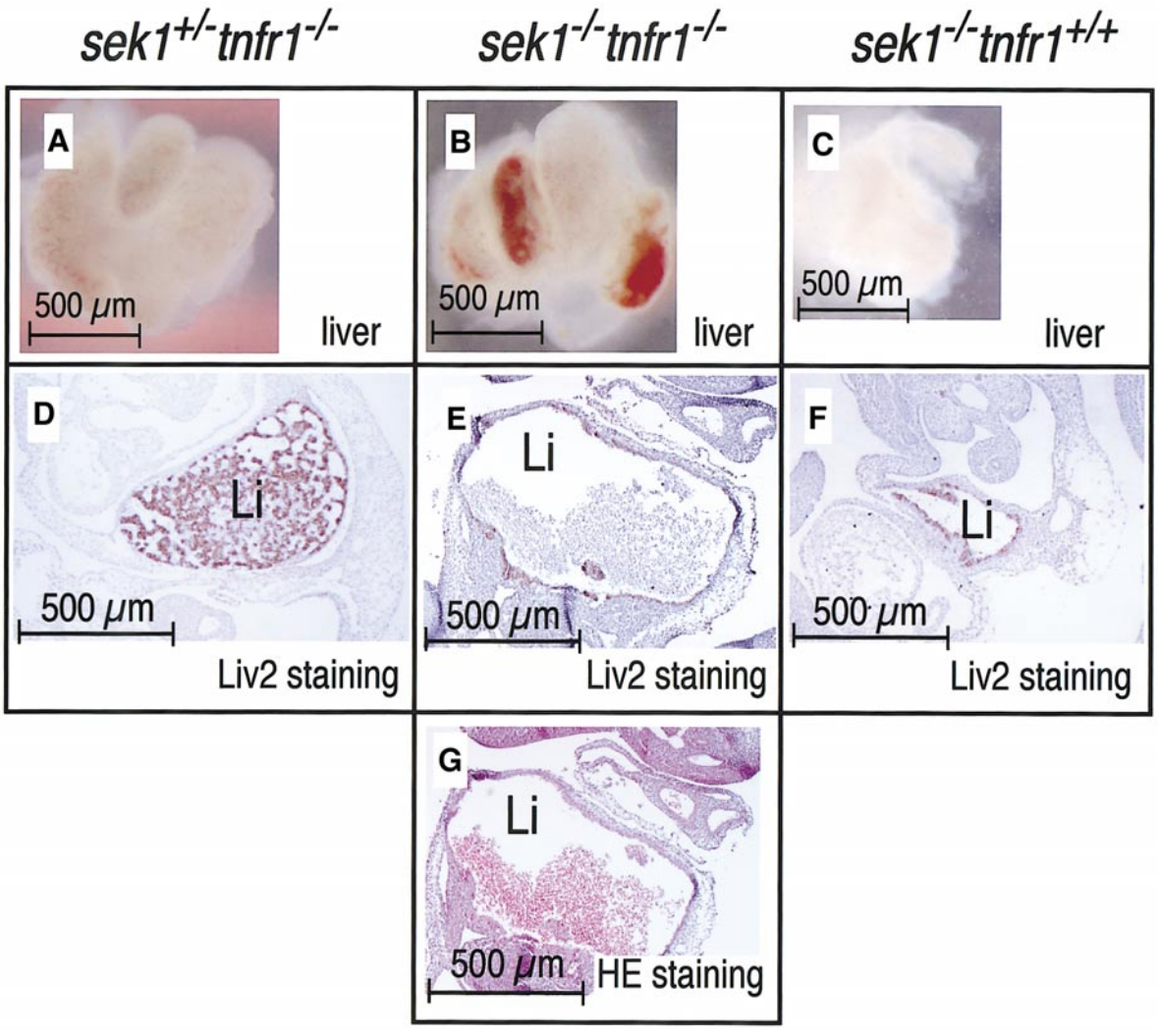


**FIG. 2.** No rescue by TNFR1 inactivation of defective liver formation in $sek1^{-/-}$ embryos. Microscopic analysis was performed in $sek^{+/-}$ $tnfr1^{-/-}$ (A, D), $sek1^{-/-}$ $tnfr1^{-/-}$ (B, E, G), and $sek1^{-/-}$ $tnfr1^{+/+}$ (C, F) fetal livers at E11.5. Transverse sections were stained with anti-Liv2 (D–F) and hematoxylin–eosin (HE; G). Li, liver. Scale bars, 500 μm.

ated as described previously (Nishina *et al.*, 1999; Johnson *et al.*, 1993; Okuda *et al.*, 1996; Pfeffer *et al.*, 1993). The genetic background of these mice used in this study was C57BL/6. For timed pregnancies, male and female mice were mated overnight, and female mice were scored for vaginal plaques in the next morning. The presence of vaginal plaques was taken to represent embryonic day 0.5 (E0.5). Animal care in our laboratory was in accordance with the guidance of University of Tokyo for animal and recombinant DNA experiments.

## RT-PCR Analysis

Each fetal liver at E10.5 and E11.5 from *sek1*$^{+/-}$ intercrosses was suspended in 100 $\mu$l of TRIzol reagent (Invitrogen), and total RNAs were prepared according to the manufacturer's instructions. First-strand cDNA was synthesized by using the Super Script II Kit (Invitrogen). Synthesized cDNAs were used as templates for PCR amplification of murine $\beta$-actin, AFP, and albumin. Samples were subjected to 30 cycles at 94°C denaturation for 30 s, 63°C annealing for 60 s, and 72°C extension for 30 s. PCR products were separated by electrophoresis on 2.5% agarose gels and stained with ethidium bromide. Primers and expected size were as described (Gualdi *et al.*, 1996): $\beta$-actin+, 5′-AAA GAC CTG TAC GCC AAC ACA GTC-3′; $\beta$-actin−, 5′-GTC ATA CTC CTG CTT GCT GAT CCA-3′ (219 bp); AFP+, 5′-CCT CCC AGT GCG TGA CGG AGA A-3′; AFP−, 5′-CAC TTC CTC CTC GGT GGC TTC C-3′ (89 bp); albumin+, 5′-CCC CAC TAG CCT CTG GCA AAA T-3′; albumin−, 5′-CTT AAA CCG ATG GGC GAT CTC ACT-3′ (127 bp).

## Production of Monoclonal Antibodies

Eight-week-old WKY/NCrj female rats were immunized with 100 $\mu$g of E11.5 murine fetal liver lysate in complete Freund's adjuvant (0.2 ml) into the hind footpads. After 20 days, the interiliac lymph nodes were removed and pooled in cold PBS as described (Sado and Okigaki, 1996). A single cell suspension was prepared from the lymph nodes by passing through 75-$\mu$m nylon mesh. The cells were washed with serum-free DMEM and fused with mouse myeloma cell line (PAI) as described (Tamatani and Miyasaka, 1990). The fused cells were plated onto a 96-well tissue culture plate at a concentration of $2 \times 10^7$ lymphomas in each plate and cultured in *h*ypoxanthine, *a*minopterin, and *t*hymidine (HAT) selection medium (Wako) containing 1× MEM NEAA (Invitrogen), 5% NCTC109 (Invitrogen), 1× HT supplement (Invitrogen), 1× aminopterin (Sigma), and IL-6 for 12 days in 5% $CO_2$ at 37°C. HAT selection medium was further added halfway into the 7th day. After HAT selection medium was replaced with fresh HT medium [GIT medium containing NEAA mixture, 5% NCTC109, HT supplement, and Hybridoma Cloning Factor (IGEN)] for 2 days, cell culture supernatants were removed from each well, and 4 plates of culture medium were altogether pooled into another 96-well plate. Each 20 $\mu$l of pooled supernatant were used for an immunohistochemical screening assay against paraffin-embedded E11.5 embryo sections. Staining was performed as described below. Positive hybridoma colonies were cloned by limiting dilution.

## Immunohistochemistry

Embryos were isolated in ice-cold PBS, fixed for 1 h in 4% paraformaldehyde at 4°C, dehydrated, and embedded in paraffin. Embryo genotypes were determined by PCR using yolk sac. Five-micrometer-thick sections were treated with 3% $H_2O_2$ in methanol for blocking the endogenous peroxidase activity. Sections were filled with 20 $\mu$l of 10% bovine serum albumin (BSA) and 0.01% NaN3 in PBS for 30 min and mixed with culture supernatant (diluted 1:1) or purified antibodies (1 $\mu$g/ml). Binding of secondary antibodies (5 $\mu$g/ml of biotinylated rabbit anti-rat IgG; VECTOR Lab; BA-4001) was detected by using biotinylated avidin-conjugated horseradish peroxidase (ABC kit; VECTOR Lab) and developed for 5 min by adding diaminobentidine (DAB). Sections were counterstained with hematoxylin and/or eosin.

For immunostaining of frozen sections, embryos were isolated in ice-cold PBS, fixed in 4% paraformaldehyde at 4°C for 1 h, cryoprotected in 10, 15, and 20% sucrose in PBS at 4°C for 4 h each, embedded in OCT compound (Miles), and frozen in liquid nitrogen. Frozen sections (10 $\mu$m) were cut on a SAKURA cryostat, transferred to poly-L-lysine-coated slides, and air dried for at 30 min at room temperature. The sections were treated with 0.3% $H_2O_2$ in PBS for blocking the endogenous peroxidase activity, filled with 20 $\mu$l of 10% BSA and 0.01% NaN3 in PBS for 30 min, and mixed with anti-hepatocyte nuclear factor (HNF)-3$\beta$ antibody (M-20, Santa Cruz Biotechnology Inc., diluted 1:100). Binding of secondary antibodies (5 $\mu$g/ml of biotinylated rabbit anti-goat IgG; VECTOR Lab; BA-5000) was detected by using biotinylated avidin-conjugated horseradish peroxidase (ABC kit; VECTOR Lab) and developed for 5 min by adding DAB. For costaining with anti-Liv2 monoclonal antibody, the above sections were further filled with 20 $\mu$l of 10% BSA and 0.01% NaN3 in PBS for 30 min and mixed with anti-Liv2 monoclonal antibody (10 $\mu$g/ml). Anti-Liv2 monoclonal antibody was detected by using anti-rat IgG-conjugated horseradish peroxidase (Jackson Laboratory) and developed for 10 min by adding DAB/$NiCl_2$.

## Whole-Mount Immunostaining

Whole embryos were collected in PBS and fixed in methanol: dimethlysulfoxide (4:1, overnight at 4°C), followed by processing for whole-mount immunostaining as described (Hogan *et al.*, 1994). The embryos were incubated with anti-Liv2 monoclonal antibody (1 $\mu$g/ml, 4°C overnight), and binding of the primary antibody was visualized by a horseradish peroxidase-labeled secondary rabbit anti-rat IgG (5 $\mu$g/ml ZYMED; 61-9520). Staining was developed by using DAB/$NiCl_2$ as a substrate. Briefly, embryos were fixed in 4% paraformaldehyde. After being dehydrated with ethanol, embryos were cleared with benzyl alcohol:benzyl benzoate (1:2).

## Cell Counting of Fetal Livers

Fetal livers obtained from *sek1*$^{+/-}$ intercrosses were isolated in ice-cold PBS. After determination of embryo genotypes by PCR, the livers were filtered through 75-$\mu$m nylon mesh in each embryo, and total cell numbers were counted. Liv2-positive cell numbers were calculated from the total liver cell numbers and the ratios of Liv2-positive cells in the paraffin section.

## Assay of BrdU Labeling

Pregnant mice were injected intravenously with 0.3 ml of 50 mg/ml BrdU (Sigma) in PBS as described (Reimold *et al.*, 2000). Embryos were isolated after 4 h from the injection and fixed for 1 h in 4% paraformaldehyde at 4°C, and genotypes of the embryos were determined by PCR. After embedding in paraffin, sections were treated with 3% $H_2O_2$ in methanol for blocking the endogenous peroxidase activity, and detection of BrdU was performed following

the instructions of the BrdU Labeling Kit (Amersham Pharmacia). The BrdU-labeled sections were also stained with anti-Liv2 antibody by the method described above.

### Assay of SAPK/JNK Activity

Wild-type livers at E10.5 (approximately $4 \times 10^5$ cells) were dissected in ice-cold PBS and put into 1.5-ml tubes at 4°C. More than four livers were pooled for one assay. Livers were incubated with anisomycine (30 $\mu$g/ml, 37°C for 30 min) or HGF (30 ng/ml at 37°C for the indicated times). The livers were suspended at 4°C in 0.5 ml of a lysis buffer consisting of 30 mM Tris–HCl, pH 7.5, 10 mM NaCl, 0.5% Nonidet P-40, 0.05% 2-mercaptoethanol, 5 mM EDTA, 0.1 mM phenylmethyl–sulphonyl fluoride, 100 $\mu$M $Na_3VO_4$, 20 $\mu$g/ml of leupeptin, 50 mM NaF, and 1 mM benzamidine. *Sek1*$^{-/-}$ E10.5 livers were isolated in ice-cold PBS and stimulated with HGF (30 ng/ml at 37°C for 15 min) in 1.5-ml tubes. The livers were suspended at 4°C in 0.1 ml of the lysis buffer and frozen by liquid nitrogen for storage at −80°C. After genotype mapping, more than four *sek1*$^{-/-}$ liver lysates were pooled for the assay. SAPK/JNK proteins were immunoprecipitated at 4°C for 2 h by using the anti-SAPK/JNK antibody (C-17, Santa Cruz Biotechnology, Inc.). The SAPK/JNK activity in the precipitated fractions was measured with GST-c-Jun as an *in vitro* substrate in the presence of 60 $\mu$M [$\gamma$-$^{32}$P]ATP as described previously (Nishina *et al.*, 1997a,b). The amounts of the precipitated SAPK/JNK that had been monitored by SDS–PAGE and immunoblotting with the anti-SAPK/JNK antibody (FL, Santa Cruz Biotechnology, Inc.) were almost constant in a series of the present experiments.

### Western Blotting

HeLa cells (approximately $1 \times 10^5$ cells) and one fetal liver from wild-type or *sek1*$^{-/-}$ embryo at E10.5 were harvested in ice-cold PBS and suspended in Leammli sample buffer (50 mM Tris–HCl, pH 6.5, 10% glycerol, 2% SDS, 0.5% 2-mercaptoethanol, and 0.02% bromophenol blue) in 1.5-ml tubes. Activated p38 was measured by SDS–PAGE and immunoblotting with anti-phosphoryrated-p38 antibody (New England Biolabs). Total amount of p38 was also monitored by SDS–PAGE and immunoblotting with the anti-p38 antibody (C-20, Santa Cruz Biotechnology Inc.).

All experiments were repeated at least three times with different batches of the cell samples, and the results were fully reproducible. Hence, most of the data shown are representative of several independent experiments.

## RESULTS

### A Novel Monoclonal Antibody, anti-Liv2, Specifically Recognizing Hepatoblasts in Murine Embryos

To investigate fetal liver development at the early stage of E9.5–E12.5 in murine embryos, we prepared novel rat monoclonal antibodies against the fetal livers at E11.5. As shown in Fig. 1, one of the antibodies, anti-Liv2, specifically recognized hepatic cells that were costained with anti-HNF-3$\beta$ antibody (Figs. 1A, 1B, and 1D) but not TER119-positive erythrocytes (data not shown). Although the nature of Liv2 antigen has not been identified yet, the cell membrane of hepatic cells was, but the cytoplasm and nuclei were not, stained with the monoclonal antibody (Fig. 1C). Almost all cells in hepatic bud at E9.5 were recognized with anti-Liv2 (Figs. 1E and 1F). Interestingly, there was a progressive decrease in the ratio of Liv2-positive to total cells as the fetal liver developed from E9.5 to E12.5 (see Fig. 3B). Thus, it is very likely that anti-Liv2 specifically recognizes so-called hepatoblasts appearing with fetal liver development. This monoclonal antibody has been applied in the present study as a useful tool to analyze hepatoblasts in various mutant mice.

### A Differential Role of SEK1 from NF-κB-Induced Anti-Apoptosis in Fetal Liver Formation

As mentioned before (see Introduction), mice lacking SEK1 or NF-κB signaling components are embryonic lethal with impaired liver formation; furthermore, both activations of SEK1 and NF-κB are induced by TNFR1 in fetal livers. Interestingly, liver apoptosis originated from the lacking of NF-κB signaling components has been rescued by the inactivation of TNFR1 (Q. Li *et al.*, 1999; Bonnard *et al.*, 2000; Rosenfeld *et al.*, 2000). Therefore, we investigated the relationship between SEK1- and TNFR1-mediated signaling pathways in whole embryos and fetal livers. C57BL/6-background *sek1*$^{-/-}$ *tnfr1*$^{-/-}$ embryos were prepared from *sek1*$^{+/-}$ *tnfr1*$^{-/-}$ intercrosses, and *sek1*$^{-/-}$ *tnfr1*$^{+/-}$ embryos were from *sek1*$^{+/-}$ *tnfr1*$^{-/-}$ and *sek1*$^{+/-}$ *tnfr1*$^{+/+}$ intercrosses (Table 1). Embryos of all three expected genotypes from *sek1*$^{+/-}$ *tnfr1*$^{-/-}$ and *sek1*$^{+/-}$ *tnfr1*$^{+/+}$ intercrosses were not present at normal Mendelian ration at E12.5 similar to those described previously (Table 1, left; Nishina *et al.*, 1999). Interestingly, embryos of all three expected genotypes from *sek1*$^{+/-}$ *tnfr1*$^{-/-}$ intercrosses were present at the normal Mendelian ration until E13.5 and became abnormal at E14.5 (Table 1, right). Embryo resorption was thus rescued by 2 days. The apparent sizes of *sek1*$^{-/-}$ *tnfr1*$^{-/-}$ embryos and livers were almost the same as those of wild-type at E11.5 (Figs. 2A and 2B). However, liver defects were not rescued in *sek1*$^{-/-}$ *tnfr1*$^{-/-}$ embryos (Figs. 2E and 2G). The SEK1-deficient livers contained capsule, hematopoietic precursors, and disorganized islands of Liv2-positive hepatoblasts (Fig. 2F), and these defects were still observed in *sek1*$^{-/-}$ *tnfr1*$^{-/-}$ livers (Figs. 2E and 2G). These results clearly indicate that embryo resorption is partially due to TNFR1-mediated signaling and that a role of SEK1 in fetal liver formation is different from that of NF-κB signaling, whose defects were rescued by inactivation of TNFR1 function.

### SEK1-Deficient Livers Showing a Decreased Number of Hepatoblast in Early Hepatogenesis

To characterize *sek1*$^{-/-}$ livers, we further measured Liv2-positive cells in *c-jun*$^{-/-}$ embryos, which are also accompanied with defective liver formation (Hilberg *et al.*, 1993;

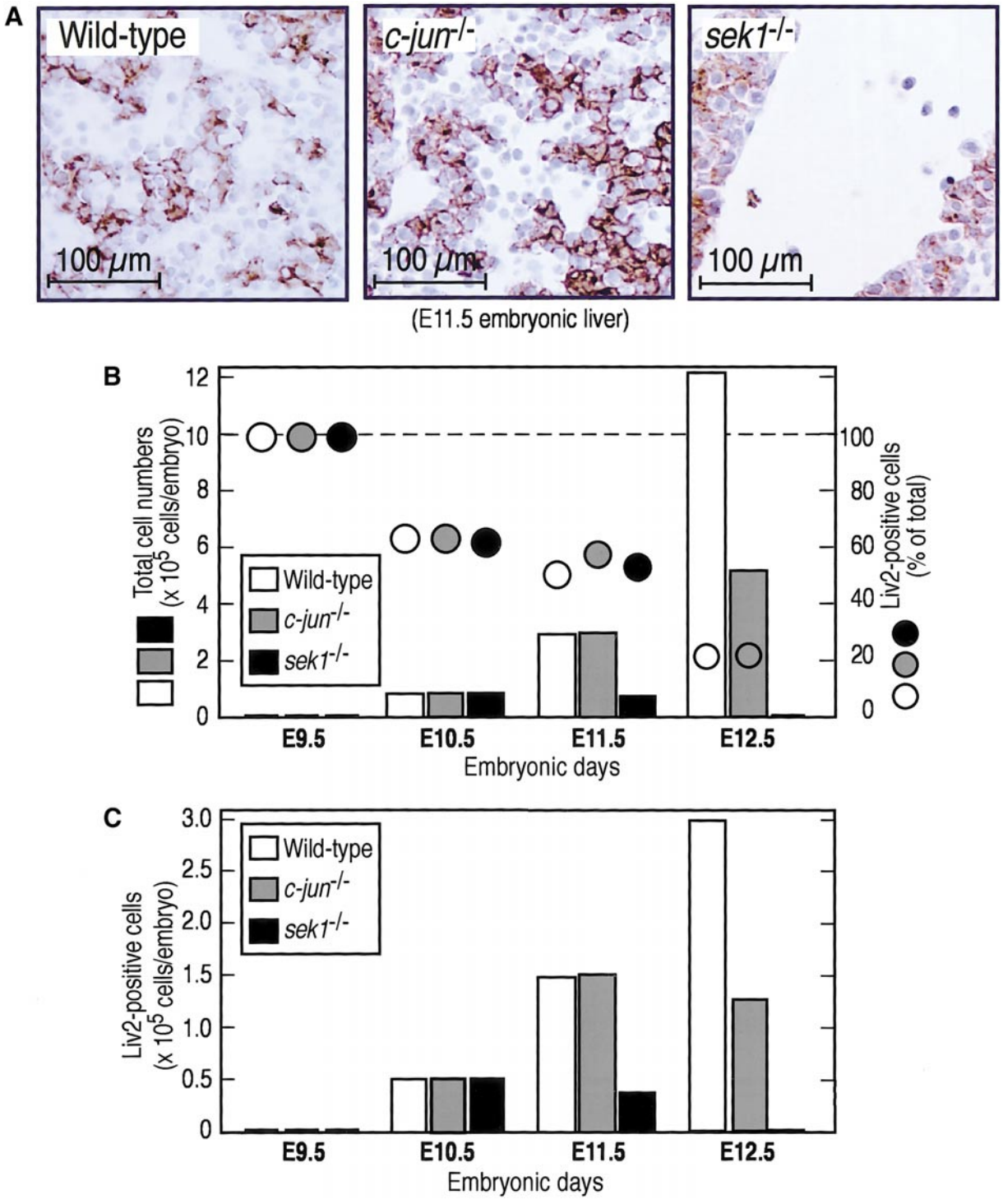


**FIG. 3.** Impaired hepatoblast development observed in *c-jun*$^{-/-}$ and *sek1*$^{-/-}$ livers. (*A*) Transverse sections of wild-type (left), *c-jun*$^{-/-}$ (middle), and *sek1*$^{-/-}$ (right) embryonic livers at E11.5 were stained with anti-Liv2. (B) Total cell numbers were estimated from cell suspension of the three embryonic livers at E9.5, E10.5, E11.5, and E12.5, and percentages of Liv2-positive cells were calculated from their sections. (C) Numbers of Liv2-positive cells in the three livers were calculated by using the total cell numbers and the percentages of Liv2-positive cells. The data shown are representative of three independent experiments.

Eferl *et al.*, 1999). As shown in Fig. 3B, total cell numbers in *sek1*$^{-/-}$ and *c-jun*$^{-/-}$ livers were significantly lower than that in wild type. However, the ratios of Liv2-positive to the total liver cells were not significantly different among the three types of mice, and they gradually decreased with liver development; approximately 100% at E9.5, 60% at E10.5, 50% at E11.5, and 20% at E12.5 (Fig. 3B). As shown in Fig. 3C, calculated Liv2-positive cells increased progressively from $5 \times 10^4$ to $3 \times 10^5$ cells during E10.5–E12.5 in wild-type fetal livers. Although the numbers of Liv2-positive cells at E10.5 were not different among wild-type, *sek1*$^{-/-}$, and *c-jun*$^{-/-}$ livers, the progressive increase observed in wild-type livers was markedly attenuated at E11.5 and E12.5 in *sek1*$^{-/-}$ and *c-jun*$^{-/-}$ livers, respectively. These results clearly indicate that impaired hepatoblast development certainly occurs in *sek1*$^{-/-}$ embryos and that *sek1* mutation is more severe than *c-jun* mutation in terms of hepatoblast development, which is consistent with the previous finding that *sek1*$^{-/-}$ embryos die more rapidly than *c-jun*$^{-/-}$ embryos.

### *SEK1-Deficient Livers Exhibiting Normal Hepatic Gene Expression*

A recent study has shown that *c-jun*$^{-/-}$ livers display normal expression of mRNAs, including those of albumin, keratin18, hepatocyte HNF-1, $\beta$-globin, and erythropoietin, some of which are putative AP-1 target genes (Eferl *et al.*, 1999). To confirm normal liver differentiation in *sek1*$^{-/-}$ embryos, the tissue-specific gene expression was measured by means of RT-PCR (Fig. 4). An early fetal hepatic marker, AFP, was expressed on the same level between wild-type and *sek1*$^{-/-}$ livers at E10.5 when cell numbers of Liv2-positive hepatoblasts were the same. Furthermore, a mature hepatic marker, albumin, was also expressed both in wild-type and *sek1*$^{-/-}$ fetal livers at E10.5 and E11.5. These results indicate that hepatic differentiation is not affected in *sek1*$^{-/-}$ fetal livers as observed in *c-jun*$^{-/-}$ ones.

### *SEK1-Deficient Livers Characterized As Impaired Cell Growth of Hepatoblasts*

We previously reported massive cell apoptosis in *sek1*$^{-/-}$ livers at E12.5 (Nishina *et al.*, 1999). This apoptosis appeared to be accompanied with the decreased number of hepatoblasts at E11.5, without significant change in the gene expression of hepatic markers (Figs. 3 and 4). Therefore, growth capacity of *sek1*$^{-/-}$ hepatoblasts at the early stage of E10.5 was analyzed by the incorporation of BrdU (Fig. 5). Interestingly, BrdU incorporation into Liv2-positive hepatoblasts was greatly reduced in *sek1*$^{-/-}$ livers compared with wild-type and *c-jun*$^{-/-}$ livers. These results suggest that *sek1* mutation may result in the impaired growth capacity of hepatoblasts at E10.5 when no other obvious defects in *sek1*$^{-/-}$ livers are observed.

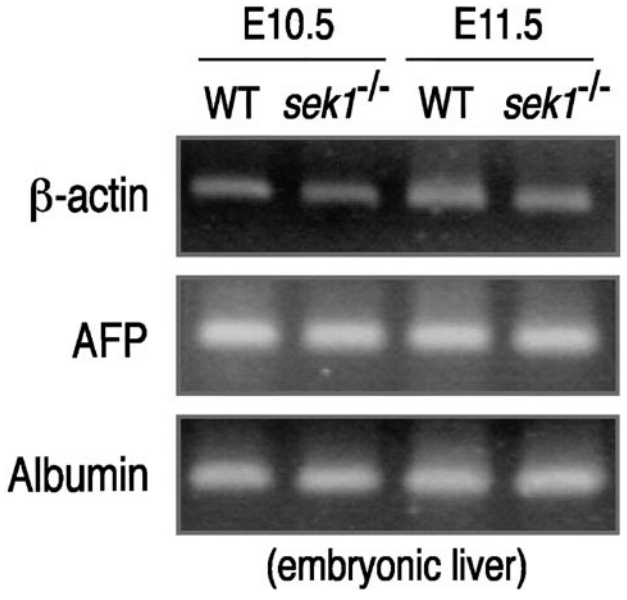


**FIG. 4.** Liver-specific gene expression normally observed in *sek1*$^{-/-}$ livers. RNAs were prepared from wild-type and *sek1*$^{-/-}$ livers at E10.5 and E11.5, and cDNA synthesis was performed as described in Materials and Methods. Gene expression of $\beta$-actin, alphafetoprotein (AFP), and albumin was analyzed by RT-PCR.

### *Genetic Interaction between sek1 and c-jun in Murine Development*

Since knockout mice of *sek1* and *c-jun* genes display the similar phenotype of impaired liver formation (Nishina *et al.*, 1999), we further examined a genetic interaction between the two genes by preparing *sek1*$^{-/-}$ *c-jun*$^{-/-}$ double mutant embryos (Table 2). According to Mendelian ratio (1:16), there was an expected number of *sek1*$^{-/-}$ *c-jun*$^{-/-}$ genotypes in E8.5 embryos, in addition to *sek1*$^{+/+}$ *c-jun*$^{+/+}$, *sek1*$^{-/-}$ *c-jun*$^{+/+}$, and *sek1*$^{+/+}$ *c-jun*$^{-/-}$ genotypes. None of *sek1*$^{-/-}$ *c-jun*$^{-/-}$ embryos were, however, observed at E10.5, though embryos of *sek1* or *c-jun* single mutant existed with expected numbers. The loss of *sek1*$^{-/-}$ *c-jun*$^{-/-}$ embryos at E10.5 appeared to be due to resorption, since all the embryos were already dead by E8.5. Thus, *sek1*$^{-/-}$ *c-jun*$^{-/-}$ double mutant embryos died before E8.5 and underwent resorption through E10.5. These results indicate that *sek1* and *c-jun* genes work synergistically during early embryonic development.

### *No Requirement of Hematopoiesis for Hepatoblast Growth in Early Hepatogenesis*

To examine a relationship between hepatoblast growth and hematopoiesis in early fetal liver, we measured the number of Liv2-positive cells at E11.5 in *AML1*$^{-/-}$ embryos, which lack definitive hematopoiesis (Fig. 6). The total numbers of hepatic cells plus blood cells in wild-type and

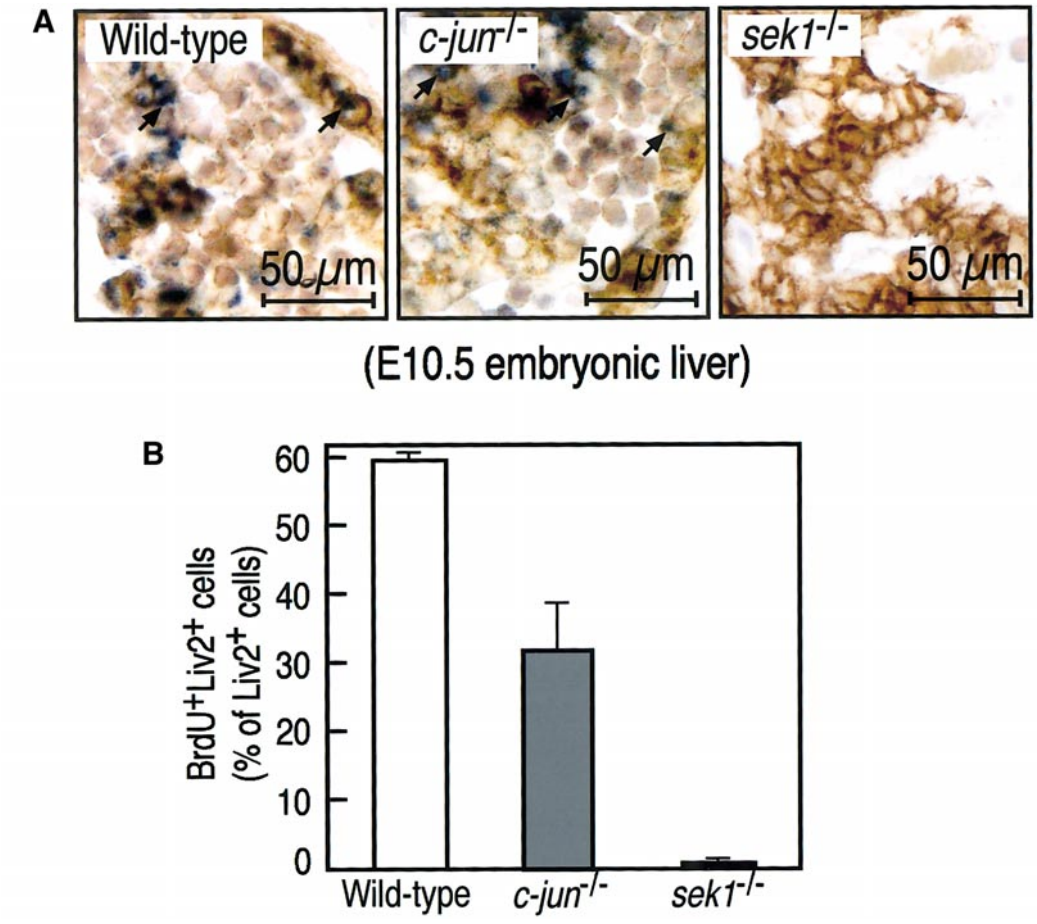


**FIG. 5.** Impaired hepatoblast growth observed in *sek1*$^{-/-}$ livers. Pregnant mice bearing E10.5 embryos were injected with 0.3 ml of 50 mg/ml BrdU. After 4 h, the embryos were isolated and fixed with 4% paraformaldehyde, and genotypes of the embryos were determined by PCR. (A) Transverse sections were prepared from the paraffin embedding of wild-type (left), *c-jun*$^{-/-}$ (middle), and *sek1*$^{-/-}$ (right) embryos and stained with anti-Liv2 (brown). Arrows indicate BrdU-incorporated (blue) Liv2-positive hepatoblasts. (B) The BrdU-incorporated cells were counted in the three embryos (wild-type, *c-jun*$^{-/-}$, and *sek1*$^{-/-}$), and the values are expressed as percentages of total Liv2-positive hepatoblasts.

*AML1*$^{-/-}$ livers at E11.5 were counted as 3.0 and 2.2 $\times$ $10^5$ cells, respectively (Fig. 6C). When paraffin sections were prepared from E11.5 fetal livers and stained with anti-Liv2 (Figs. 6A and 6B) or anti-TER119 (data not shown), there were 44 and 79% Liv2-positive cells in wild-type and *AML1*$^{-/-}$ livers, respectively (Fig. 6D). *AML1*$^{-/-}$ fetal livers at E11.5 contained approximately 80% Liv2-positive cells and 20% TER119-positive primitive erythrocytes. There-

**TABLE 2**

Analysis of Embryos Obtained from *sek1*$^{+/-}$ *c-jun*$^{+/-}$ Intercrosses

| Embryonic stage | *sek1*$^{+/+}$ *c-jun*$^{+/+}$ | *sek1*$^{+/+}$ *c-jun*$^{+/-}$ | *sek1*$^{+/-}$ *c-jun*$^{+/+}$ | *sek1*$^{+/-}$ *c-jun*$^{+/-}$ | *sek1*$^{-/-}$ *c-jun*$^{+/+}$ | *sek1*$^{-/-}$ *c-jun*$^{+/-}$ | *sek1*$^{+/+}$ *c-jun*$^{-/-}$ | *sek1*$^{+/-}$ *c-jun*$^{-/-}$ | *sek1*$^{-/-}$ *c-jun*$^{-/-}$ | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| E8.5 | 2 | 4 | 3 | 16 | 1 | 3 | 2 | 4 | 2 (2) | 37 |
| E10.5 | 6 | 12 | 10 | 24 (1) | 7 (2) | 10 (3) | 5 | 10 (1) | 0 | 84 |

*Note.* Embryos were isolated at the indicated time points of gestation and analyzed for viability. Genotypes of embryos were determined by PCR, and the numbers of each genotype are listed below. Dead embryos were defined as the states of hearts that stopped beating, and the beating was monitored by using inverted microscopy. The numbers in parentheses indicate dead embryos.

fore, Liv2-positive cells were calculated as 1.3 and $1.7 \times 10^5$ in wild-type and *AML1*$^{-/-}$ livers, respectively (Fig. 6E). Thus, the cell number of hepatoblasts was rather increased in *AML1*$^{-/-}$ livers than in wild-type ones. These results indicate that hematopoiesis and growth factors from blood cells are not essentially required for hepatoblast growth in early hepatogenesis.

### *Impairment of HGF-Induced SAPK/JNK Activation in Fetal Livers Lacking SEK1*

To elucidate the biochemical interaction between SEK1 and SAPK/JNK, we first investigated whether HGF, which is produced from the nonblood cells, can activate SAPK/JNK in fetal liver cells expressing HGF receptor, c-Met. In a previous report (Nishina *et al.*, 1999), we could not detect any SAPK/JNK activation by HGF in primary cell culture prepared from fetal livers. Therefore, in this study, we screened the intact conditions of liver cells and found that strong SAPK/JNK activation was observed in response to HGF by using whole livers at E10.5, where about 60% of cells are Liv2-positive hepatoblasts. As shown in Fig. 7, there was maximally more than a 25-fold increase in SAPK/JNK activity at 10 min, and the activity decreased rapidly (Fig. 7A). Such marked activation of SAPK/JNK was, however, greatly reduced in *sek1*$^{-/-}$ fetal livers (Fig. 7B). Interestingly, another member of stress-activated MAP kinases, p38, was constitutively phosphorylated without stimulation by HGF in fetal livers, and this phosphorylation was still observed in *sek1*$^{-/-}$ fetal livers (Fig. 7C). These results indicate that SEK1 is required for HGF-induced full activation of SAPK/JNK but not for p38 activation and that HGF is one of growth factors regulating hepatoblast growth in early fetal liver development.

## DISCUSSION

Embryonic liver formation is genetically a crucial checkpoint in fetal hematopoiesis and development. Although hematopoiesis has been characterized at cellular and molecular levels, hepatogenesis and liver formation are just beginning to be characterized. To analyze the early liver development, we first screened monoclonal antibodies, specifically, recognized murine fetal livers, by using transverse sections of E11.5 embryos. One of the antibodies, anti-Liv2, is applicable to paraffin sections and whole mount embryos, and Liv2 antigen appears to be localized in the cell membrane (Fig. 1). The ratios of Liv2-positive cells from E9.5 to E12.5 are consistent with those of hepatic cells defined as hepatoblasts (Fig. 3) (Grisham and Thorgeirsson, 1997). In this study, we measured exact cell numbers of hepatoblasts in the fetal liver development of wild-type, *sek1*$^{-/-}$, and *c-jun*$^{-/-}$ mice (Fig. 3). Thus, our present experiments proved anti-Liv2 to be a useful tool for identifying murine hepatoblasts in early fetal livers, though the Liv2 antigen and its physiological role have not been determined yet.

Previously, we and another group reported that *sek1*$^{-/-}$ embryos die between E10.5 and E12.5 with the decreased number of cytokeratin-positive hepatocytes at E11.5 and massive hepatocyte apoptosis at E12.5 (Ganiatsas *et al.*, 1998; Nishina *et al.*, 1999). To extend the above results, we examined an interesting question of whether hepatic apoptosis and embryonic lethality observed in *sek1*$^{-/-}$ mice are rescued by introduction of *tnfr1* gene mutation (Table 1; and Fig. 2). As shown in Fig. 8, TNFR1 relays TNF$\alpha$ stimulation to three separated pathways, which include the induction of apoptosis, NF-$\kappa$B activation, and SAPK/JNK activation (Liu *et al.*, 1996). Activation of NF-$\kappa$B protects against TNF$\alpha$-induced apoptosis. Therefore, mice lacking components of NF-$\kappa$B signaling pathway, such as RelA, IKK2, NEMO, or T2K, are embryonic lethal with massive liver apoptosis and are rescued by the introduction of *tnfr1* mutation. On the other hand, a physiological role of SEK1 and/or MKK7-mediated SAPK/JNK activation in response to TNFR1 stimulation remains to be resolved. In the present study, we examined the phenotype of body sizes and fetal livers of both SEK1 and TNFR1 double knockout mice. Interestingly, embryo resorption is partially inhibited in *sek1*$^{-/-}$ *tnfr1*$^{-/-}$ mice; however, liver defect by *sek1* mutation is not rescued (Table 1; and Fig. 2). These results clearly show that SEK1-mediated signal plays an important role apparently different from NF-$\kappa$B signal in fetal liver formation. Another important conclusion from the results of *sek1*$^{-/-}$ *tnfr1*$^{-/-}$ mice is that TNFR1 plays a role in embryo resorption. Some studies suggest the involvement of TNF$\alpha$ in embryo loss and resorption by showing its expression (Gendron *et al.*, 1990; Haddad *et al.*, 1997; Lea *et al.*, 1998). Our genetic experiments showing partial rescue of embryo resorption by inactivation of TNFR1 function consist of the idea that TNFR1-mediated death signaling plays a role in a clearance of abnormal embryos. Thus, TNF$\alpha$ elicits a wide spectrum of cellular responses, including apoptosis and cell growth. TNFR1 may relay its stimulation to SEK1-mediated cell growth in hepatoblasts, resulting in cell survival. Balance of three separated pathways may be important to elicit various cellular responses, depending on the type of cell and its developmental stage (Fig. 8).

To find the primary cause of liver defect by *sek1* mutation, we analyzed *sek1*$^{-/-}$ livers using anti-Liv2 and found the decreased number of hepatoblasts in the mutant embryos at E11.5 (Fig. 3). Furthermore, we found impaired BrdU incorporation into Liv2-positive hepatoblasts in *sek1*$^{-/-}$ livers at E10.5 (Fig. 5). Since liver-specific gene expression was normal in *sek1*$^{-/-}$ fetal livers (Fig. 4), the liver defects in *sek1*$^{-/-}$ embryos are very likely due to the impaired cell growth of hepatoblasts in early hepatogenesis, resulting in decreased number of hepatoblasts and massive hepatoblast apoptosis. A recent report showing maturation of SEK1-null ES cells into hepatic lineage *in vitro* also indicates that the liver defect by *sek1* mutation is not due to impaired differentiation or maturation of hepatocytes

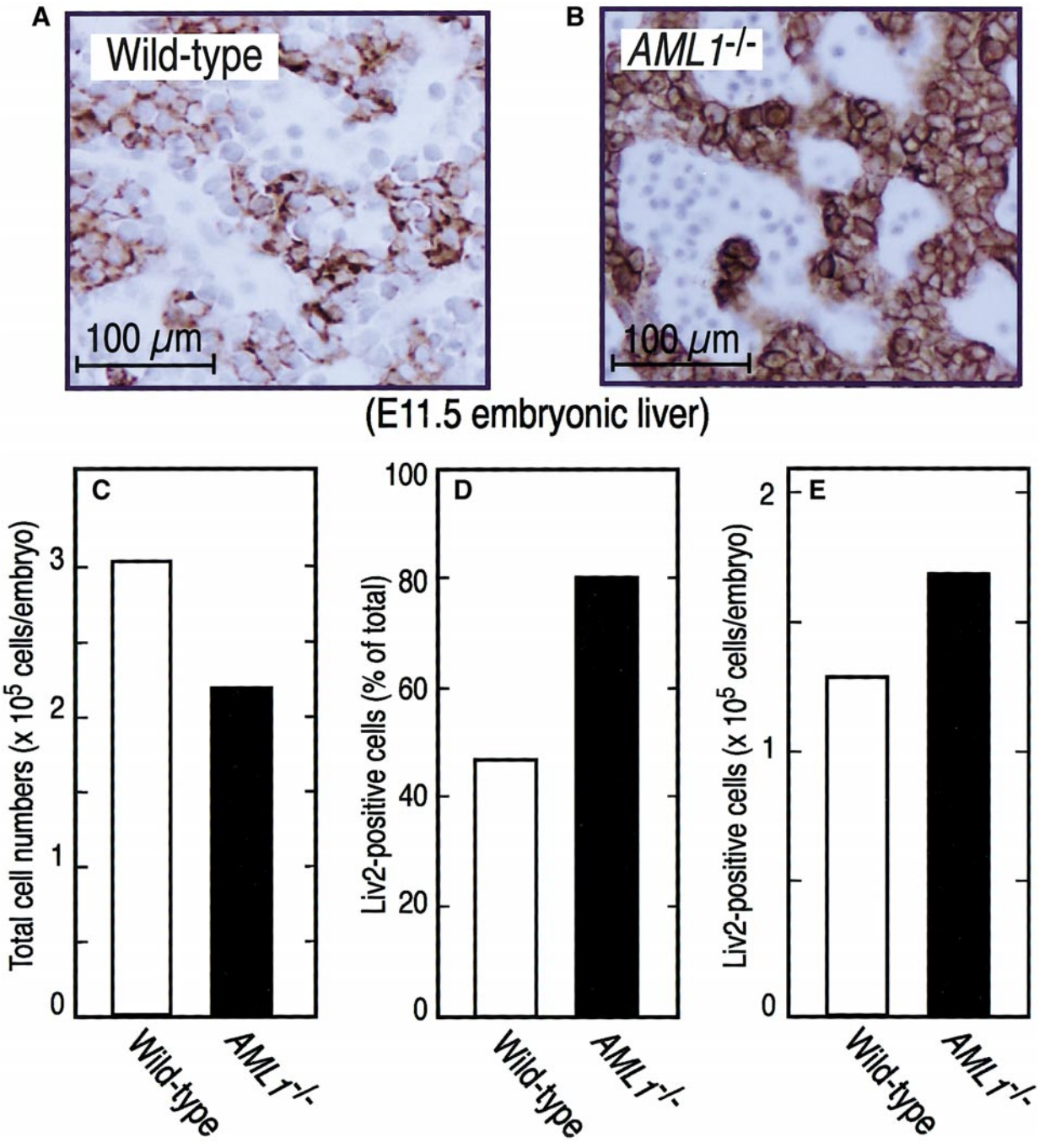


**FIG. 6.** No impairment of hepatoblast development in *AML1*$^{-/-}$ embryos that lack definitive hematopoiesis. Transverse sections of wild-type (A) and *AML1*$^{-/-}$ (B) embryonic livers at E11.5 were stained with anti-Liv2. Total cell numbers (C), percentages of Liv2-positive cells (D), and numbers of Liv2-positive cells (E) in the two livers were estimated as shown in Fig. 3. The data shown are representative of three independent experiments.

(Hamazaki *et al.*, 2001). Thus, SEK1 may provide crucial and specific growth and survival signals for hepatoblasts.

Interestingly, results that impaired SAPK/JNK activation in *sek1*$^{-/-}$ fetal livers extend our recent report that synergistic activation of SAPK/JNK is impaired in SEK1-deficient murine embryonic stem cells and MKK7-deficient murine mast cells (Wada *et al.*, 2001; Sasaki *et al.*, 2001). Furthermore, mice lacking MKK7 are also embryonic lethal be-

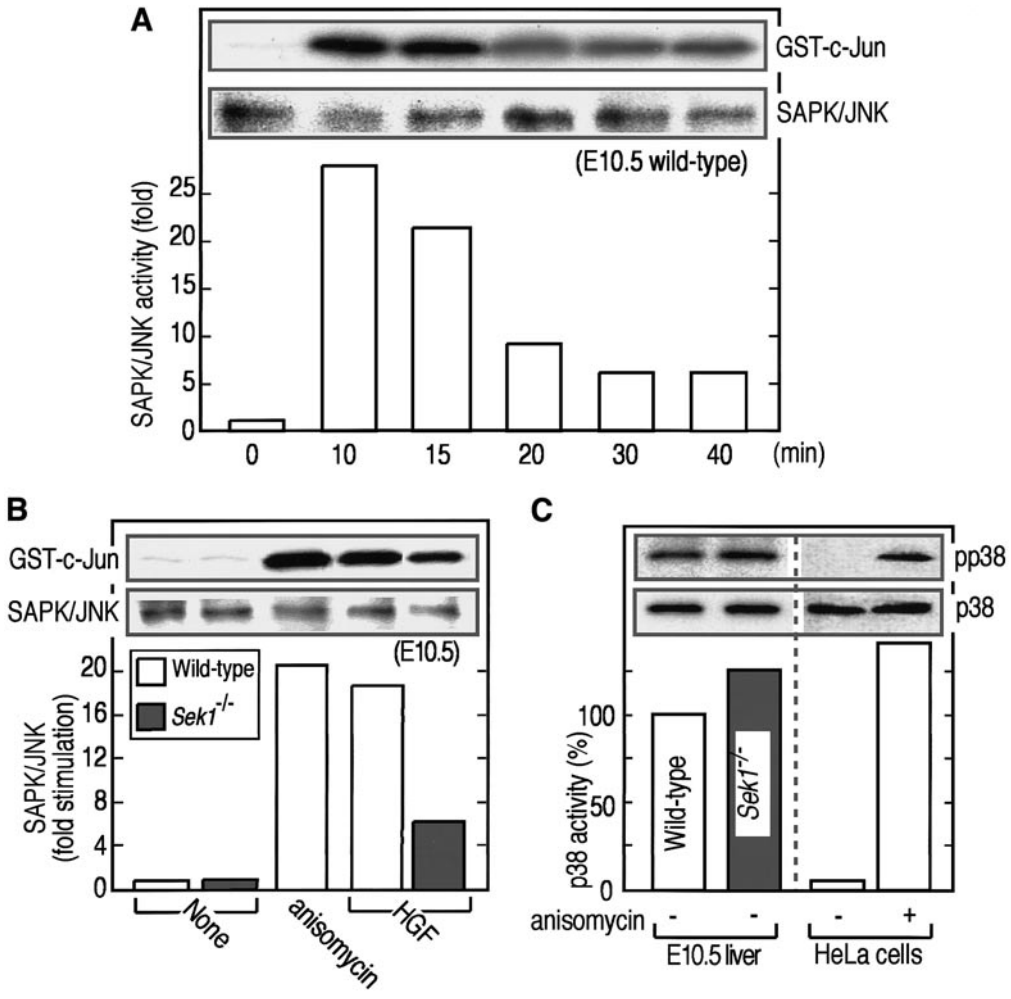


**FIG. 7.** HGF-induced activation of SAPK/JNK in wild-type fetal livers and its impairment in *sek1*$^{-/-}$ livers. (A) Cell suspension of four fetal livers (approximately $4 \times 10^5$ cells) isolated from wild-type embryos at E10.5 was incubated with 30 ng/ml of HGF at 37°C for the indicated times. SAPK/JNK was immunoprecipitated, and the activity was measured with GST-c-Jun as a substrate in the presence of [$\gamma$-$^{32}$P]ATP (insets). The activities are expressed as the fold stimulation compared with the control level observed without HGF. (*B*) Embryos at E10.5 from *sek1*$^{+/-}$ intercrosses were isolated, and their genotypes were later determined by PCR. Each liver was isolated, individually incubated with 30 $\mu$g/ml of anisomycine or 30 ng/ml of HGF at 37°C for 15 min, and rapidly frozen in liquid $N_2$ for storage at −80°C. After determination of the genotypes, *sek1*$^{+/+}$- and *sek1*$^{-/-}$-matched four fetal livers were pooled and subjected to SAPK/JNK assay as shown in (A). The activities are expressed as the fold stimulation compared with the control level observed without HGF in wild-type fetal livers. (C) The wild-type and *sek1*$^{-/-}$ fetal livers were also prepared, and phosphorylated levels of p38 were measured with an anti-phospho-p38 antibody. As control experiments, HeLa cells were treated with 30 $\mu$g/ml of anisomycine at 37°C for 15 min, and the phosphorylation of p38 was analyzed. The p38 phosphorylation levels are expressed as percentages of the level observed in *sek1*$^{+/+}$ fetal livers.

tween E11.5 and E12.5 with impaired liver formation and a decreased level of SAPK/JNK activation (T. Wada *et al.*, unpublished data). Thus, synergistic activation of SAPK/JNK by SEK1 and MKK7 seems to occur in fetal livers and to be crucial for hepatoblast growth in mouse development (Fig. 8). SEK1 also phosphorylates and activates p38 MAP kinase *in vitro* and in SEK1-overexpressed mammalian cells (Derijard *et al.*, 1995; Lin *et al.*, 1995), and p38 is indicated to be involved in the growth regulation of fetal liver (Awad *et al.*, 2000). In the present study, we found highly phosphorylated p38 in both wild-type and *sek1*$^{-/-}$ fetal livers at E10.5 (Fig. 7), indicating that SEK1 does not regulate phosphorylation of p38 in fetal liver cells.

Since c-Jun is one of the target molecules in SEK1-mediated SAPK/JNK signaling, liver defects in *c-jun*$^{-/-}$ embryos have attracted considerable attention. A recent study reported that there is reduced cell growth and increased apoptosis in *c-jun*$^{-/-}$ fetal hepatocytes and fibro-

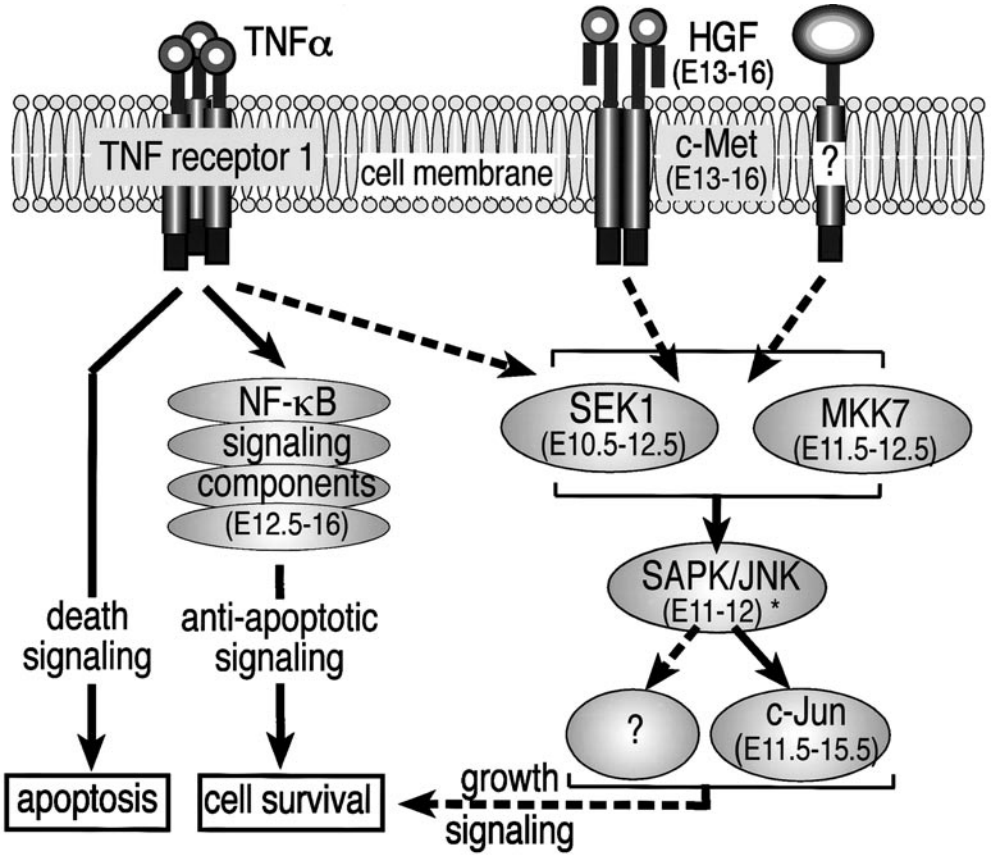


**FIG. 8.** A proposed model for SEK1-associated signaling pathways in hepatoblasts. The numbers in parentheses are dates of embryonic lethality reported in previous papers and this study. Solid and broken lines show signaling pathways reported in previous and the present studies, respectively. See Discussion for the explanation. Asterisk (*), *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ mutant mice die between the 11th and 12th days of gestation (Kuan *et al.*, 1999).

blasts at E12.5 (Eferl *et al.*, 1999). One mechanism by which lack of c-Jun could result in impaired proliferation was also reported as below. The proliferation defect in *c-jun*$^{-/-}$ fibroblast cell lines was found to be p53-dependent, indicating that the alterations of proliferation, and probably also the increased propensity of cells to undergo apoptosis, may involve p53-dependent pathways (Schreiber *et al.*, 1999). Our present experiments clearly showed that liver defects were, in terms of hepatoblast growth, much more severe in *sek1*$^{-/-}$ embryos than in *c-jun*$^{-/-}$ ones (Figs. 3 and 5). These results indicate that SEK1-mediated SAPK/JNK activation regulates another target molecule(s) in addition to c-Jun in fetal liver cells (Fig. 8). Actually, the phosphorylation of c-Jun does not appear to be crucial in fetal liver formation, since mice harboring a mutant allele of c-Jun with serines 63 and 73 mutated to alanines (*JunAA*) are healthy and fertile; histological examination of several organs, including the liver, reveals no obvious abnormalities (Behrens *et al.*, 1999). Thus, c-Jun protein itself, but not the phosphorylation of serines 63 and 73 by SAPK/JNK, is essential for fetal liver formation. However, our present experiments showed that *sek1*$^{-/-}$ *c-jun*$^{-/-}$ double mutant mice died very early in embryogenesis before E8.5 (Table 2). These results indicate that SEK1-mediated SAPK/JNK activation actually regulates c-Jun and is crucial during the mammalian embryogenesis.

Furthermore, we examined a relationship between early hepatogenesis and hematopoiesis because two recent papers reported pivotal interactions between hepatogenesis and hematopoiesis in late fetal liver development. One paper showed that OSM, an IL-6 family cytokine, in combination with glucocorticoid, induces maturation of hepatocytes at E14.5, suggesting a paracrine mechanism of hepatogenesis by blood cells (Kamiya *et al.*, 1999). Another paper reported that primary culture of fetal hepatic cells from murine embryos at E14.5 supports expansion of bloods cells, giving rise to myeliod, lymphoid, and erythroid lineages, and furthermore, in turn, promotion of hepatic development by OSM and glucocorticoid strongly suppressed *in vitro* hematopoiesis (Kinoshita *et al.*, 1999). In the present study, we

found that the size of *AML1*$^{-/-}$ fetal livers at E11.5, which lack definitive hematopoiesis, was smaller compared with that of wild-type; however, a cell number of Liv2-positive hepatoblasts was rather higher in *AML1*$^{-/-}$ fetal livers than in wild-type ones (Fig. 6). These results indicate that hepatoblast development occurs in a region derived from the foregut endoderm without definitive hematopoiesis and provides an idea that there is a place where blood cells can expand and differentiate in early fetal liver development.

As described above, hepatoblast development in early hepatogenesis does not require definitive hematopoiesis. Therefore, growth factor(s), which produced from nonblood cells, together with its receptor(s) on hepatoblasts, could relay its stimulation to SEK1 activation and promoting cell growth and cell survival (Fig. 8). This idea is supported by a recent report showing that vasculogenic endothelial cells and nascent vessels are critical for the earliest stages of hepatogenesis, prior to blood vessel function (Matsumoto *et al.*, 2001). Various growth factors, such as HGF, epidermal growth factor, IL-1, and TNF$\alpha$, have been implicated in hepatogenesis (Grisham and Thorgeirsson, 1997). However, mice lacking the TNF$\alpha$, TNFR1, IL-1, or IL-1R genes did not have any defects in liver formation (Mittrucker *et al.*, 1996). By contrast, HGF-deficient mice die between E13 and E16 with liver failure, and the embryonic livers are reduced in size and show extensive loss of hepatocytes (Schmidt *et al.*, 1995; Uehara *et al.*, 1995). As shown in Fig. 7, we found that HGF is capable of stimulating SAPK/JNK activity in wild-type fetal livers at E10.5 and that the activation is markedly impaired in *sek1*$^{-/-}$ fetal livers. These results provide the first biochemical linkage between HGF and SEK1-mediated SAPK/JNK signaling in murine fetal livers. Thus, HGF receptor, c-Met, is one of the candidates for relaying the SEK1 activation and promoting hepatoblast growth and survival. However, *hgf*$^{-/-}$ mice die later than *sek1*$^{-/-}$ mice and have an additional defect in placental development (Schmidt *et al.*, 1995; Uehara *et al.*, 1995). Therefore, another factor(s), which could also induce SEK1 activation, may be more essential for hepatoblast growth in early hepatogenesis (Fig. 8). Thus, SEK1 may receive various signals from cell-surface receptors to regulate hepatoblast growth and survival in murine embryogenesis.

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