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Mice lacking Dok-1, Dok-2, and Dok-3 succumb to aggressive histiocytic sarcoma

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Histiocytic sarcoma (HS), a rare hematological malignancy, is an aggressive neoplasm that responds poorly to therapy. The molecular etiology and pathology of this disease remain unclear, hampering the development of an effective therapy, and there remains a need for more, and more realistic, animal models. HS cells typically show a histiocytic (ie, tissue macrophage-like) morphology and express histiocyte/macrophage markers in the absence of lymphocyte markers. In this study, we report that *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice develop HS, but do not exhibit elevated incidence of other types of tumors. These mutant mice showed earlier mortality than wild-type (WT) or the other mutant mice, and this mortality was associated with HS. In total, 17 of 21 tumor-bearing *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice necropsied at 25–66 weeks of age showed multiple organ spread, with osteolytic lesions and orthotopic invasion from the bone marrow to skeletal muscle. Tumors from the mice were transplantable. In addition, all *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice, but only a small proportion of *Dok-3*$^{-/-}$ mice and no *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$ mice, exhibited abnormal accumulation of macrophages in the lung on necropsy at 8–12 weeks of age. Macrophages derived from *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice displayed an exaggerated proliferative response to macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) compared with WT and mutant controls. Together, these findings indicate that Dok-1, Dok-2, and Dok-3 cooperatively suppress aggressive HS, and commend *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice as a useful model for the study of this neoplasia.

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Histiocytic sarcoma (HS) is a malignant proliferation of cells showing morphological and immunophenotypic features of mature histiocytes, which represent tissue-resident macrophages.[1,2] Until recently, HS, which was also known as malignant histiocytosis, was often confused with anaplastic large B-cell lymphoma or with other malignant lymphomas. However, it has been established that true HS is a distinct and rare disease that is only about 0.1% as frequent as malignant lymphomas, which can be identified by the presence of B- or T-cell markers and/or CD30.[2] By definition, HS is negative for lymphocyte markers, but positive for histiocyte/macrophage markers such as CD163 in humans.[1,2] The tumor comprises a diffuse noncohesive proliferation of large cells, round to oval in shape, with abundant, eosinophilic cytoplasm and nuclear atypia. HS is an aggressive neoplasm with most patients dying of progressive disease. Patients may present with a solitary mass of HS, which is predictive of a relatively favorable outcome, but some patients show a systemic pattern of tumor spread.[1,2] As the molecular etiology of this disease is unknown, the development of rational therapeutics has been difficult. Although the generation of animal models is an essential step for the study of etiology, the paucity of mouse models that represent aggressive HS has remained an outstanding problem.

We and others previously identified Dok-1 as a common substrate of protein-tyrosine kinases (PTKs) including Bcr-Abl, a cause of chronic myelogenous leukemia,[3,4] and we further demonstrated that Dok-1 is a negative regulator of

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PTK-mediated proliferation and transformation of cells.[5,6] Indeed, mice lacking both Dok-1 and its closest homolog Dok-2 developed a myeloproliferative disorder with a non-aggressive phenotype.[7,8] It is believed that Dok-1 and Dok-2 have virtually identical roles in myeloid lineages.[7–9]

The Dok family consists of seven members, Dok-1 to Dok-7, which share structural similarities characterized by the NH_2-terminal pleckstrin homology and phosphotyrosine-binding domains, followed by the src homology 2 target motifs in the COOH-terminal moiety, suggesting an adaptor function.[3,4,10–14] Among these members, only Dok-1, Dok-2, and Dok-3 are preferentially expressed in hematopoietic cells, or myeloid cells in particular, and comprise a closely related subgroup with regard to primary structure.[7,9,15] Similar to Dok-1 and Dok-2, Dok-3 is also a negative regulator of PTK-mediated signaling, despite being a relatively distant member of this subgroup.[11,16–19] However, mice lacking Dok-3 alone or Dok-1 and its closest homolog Dok-2 in combination do not develop aggressive tumors of hematopoietic cells.[7,8,18] In this study, we demonstrate that mice lacking Dok-1, Dok-2, and Dok-3 provide a model system for aggressive HS. Triple null mutant mice showed an early lethal phenotype that is associated with HS, and the tumor, which was transplantable, showed multiple organ invasion.

MATERIALS AND METHODS

Mice

Dok-1$^{-/-}$*Dok-2*$^{-/-}$ and *Dok-3*$^{-/-}$ mice were generated as previously described.[7,20] *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ (TKO) mice were obtained by crossing *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$ and *Dok-3*$^{-/-}$ mice (Supplementary Figure S1). Mice were genotyped by a standard PCR using DNA isolated from the tail tips. Primers to amplify the wild-type (WT) and targeted *dok-1*, *dok-2*, and *dok-3* loci have been previously described.[7,20] All mice were maintained in a mixed genetic background of strains 129/SvJ and C57BL/6 under pathogen-free conditions in the animal care facilities at Tokyo Medical and Dental University and The University of Tokyo. The experimental protocols have been approved by the animal ethics committees of the two institutions.

Histological Analysis

For hematoxylin and eosin (H&E) staining, tissue samples, aside from bones, were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and stained. Bones were fixed similarly and decalcified in 14% EDTA solution for 3 days at room temperature with gentle stirring before staining with H&E. For immunohistochemistry using antibodies to Mac-2, PCNA, and Ki-67, paraffin sections were processed with 10 mM citrate buffer (pH 6.0) in a microwave (95°C, 15 min) and subjected to standard immunohistochemical staining using the streptavidin–biotin–peroxidase complex method. For immunohistochemistry using antibodies to F4/80, CD68, CD3, and B220, cryostat sections were fixed in 95% acetone at 4°C for 20 min and were also subjected to standard immunohistochemical staining using the streptavidin–biotin–peroxidase complex method. The slides were counterstained with hematoxylin. Endogenous peroxidase activity was inactivated by incubation with 3% hydrogen peroxide in methanol (at room temperature for 15 min). The following antibodies were used: anti-Mac-2 rat monoclonal antibody (M3/38, Cedarlane, Burlington, ON, Canada; dilution 1:400); anti-F4/80 rat monoclonal antibody (BM8, BD Biosciences, San Diego, CA, USA; dilution 1:100); anti-CD68 rat monoclonal antibody (FA-11, Serotec, Kidlington, UK; dilution 1:100); anti-B220 rat monoclonal antibody (RA3-6B2, BD Biosciences; dilution 1:100); anti-CD3 hamster monoclonal antibody (145–2C11, BD Biosciences; dilution 1:100); anti-PCNA mouse monoclonal antibody (PC10, Dako, Glostrup, Denmark; dilution 1:100); and anti-Ki-67 rat monoclonal antibody (TEC-3, Dako; dilution 1:50). Only nonautolyzed tissues from moribund or recently deceased mice were subjected to histological analysis.

Microscopy

Sections were viewed using an AX80 microscope (Olympus, Tokyo, Japan) with either a × 20 or × 40 PlanApo objective. Images were captured using a DP70 digital camera and DP Controller software (Olympus).

Transplantation

Nucleated bone marrow cells or splenocytes (2×10^6 cells) prepared from the donor were intravenously injected into lethally irradiated WT recipient mice. Irradiation was performed using an IBL-437C instrument (^{137}Cs, CIS Bio-International, Gif-sur-Yvette, France) at 9 Gy, and no survival was observed beyond 2 weeks after irradiation in the absence of transplantation. In Figure 3, TKO and WT mice at 78–88 weeks of age were used as donors and WT mice at 8–16 weeks of age were used as recipients. In Figure 4c, TKO and WT mice at 6–10 weeks of age were used as donors and WT mice at 8–16 weeks of age were used as recipients: these recipient mice were killed 8–10 weeks after transplantation and histological studies were performed.

Bone Marrow-Derived Macrophages

Bone marrow cells were cultured in DME medium containing 100 ng/ml of recombinant murine macrophage colony-stimulating factor (M-CSF, PeproTech, Rocky Hill, NJ, USA) and 15% FCS. After 7 days of culture, adherent cells were maintained in the absence of M-CSF for 16 h and were used as bone marrow-derived macrophages.

Cell Viability Assay

Bone marrow-derived macrophages (1×10^4) were plated in 96-well plates in quadruplicate, cultured in RPMI-1640 medium containing 15% FCS in the presence or absence of M-CSF or recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF, PeproTech) for 5 days.

Cells were then treated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, St Louis, MO, USA) for 4 h at 37°C, cellular formazan product was dissolved with acidic isopropanol, and the absorbance at 570 nm was measured spectrophotometrically to evaluate viable cells using an Emax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

RESULTS

TKO Mice Succumb to HS

To examine whether Dok-1, Dok-2, and Dok-3 cooperatively suppress malignant tumor formation *in vivo*, we generated TKO mice. These mice were born at the expected Mendelian frequency without any abnormality in appearance evident by visual inspection. However, nearly half (15 of 33) of the TKO mice died between 14–51 weeks after birth, whereas all WT and *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$ mice as well as all *Dok-3*$^{-/-}$ mice but one remained alive (Figure 1a). To gain insight into the early lethal phenotype of TKO mice, we first performed conventional histological studies. H&E staining of tissue sections revealed that TKO mice, but neither the other mutants nor the WT controls, showed a markedly high incidence (24 of 41) of large cell tumors at < 65 weeks of age, characterized by the accumulation of abnormal cells with histiocytic morphology in the bone marrow, spleen, and/or liver (Figure 1b). The aberrant cells are distinguished morphologically from other hematopoietic cells by their round shape, prominent nuclear atypia, and eosinophilic cytoplasm, features that

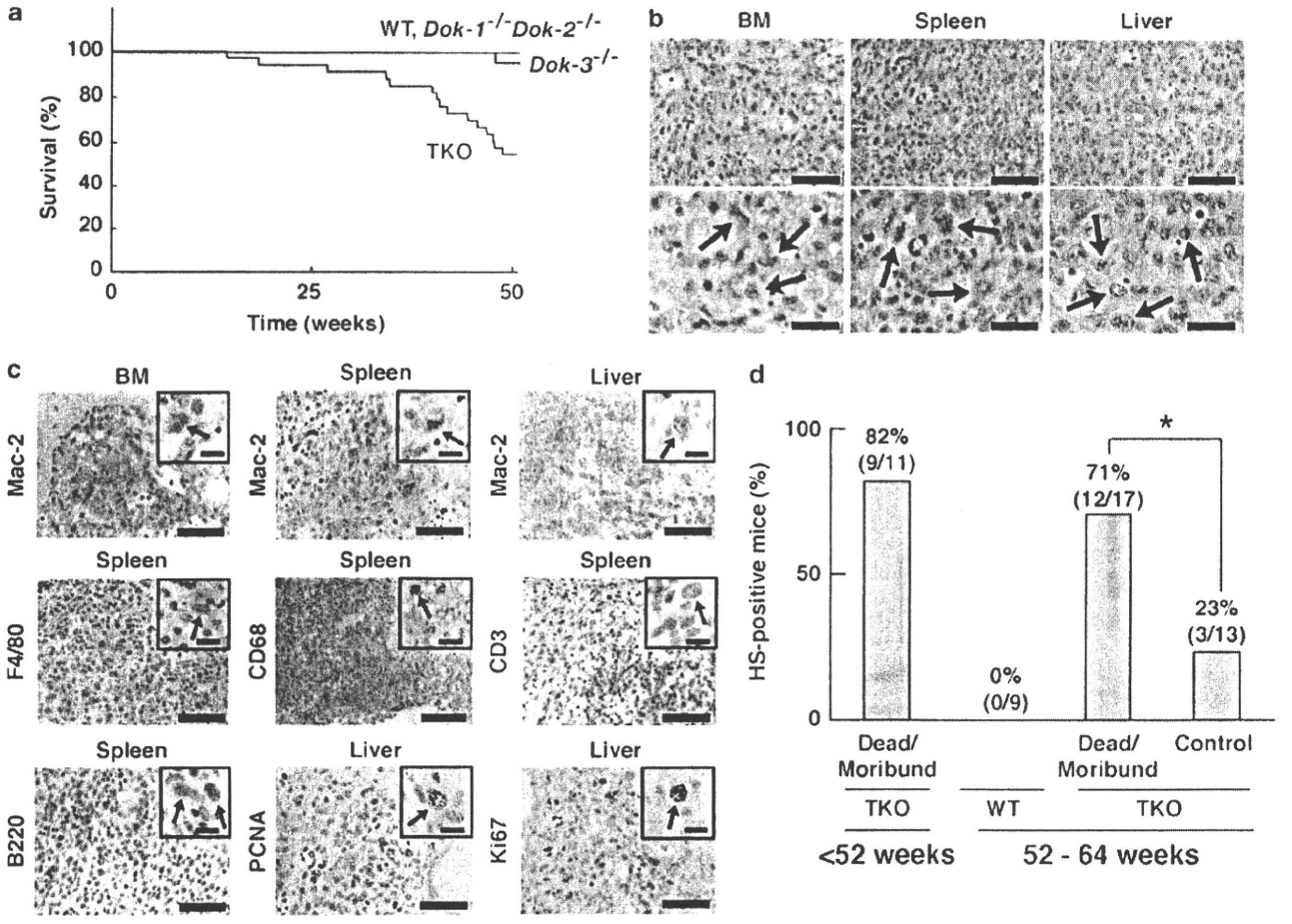

Figure 1 TKO mice develop HS with early mortality. (**a**) Survival of TKO ($n = 33$), *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$ ($n = 20$), *Dok-3*$^{-/-}$ ($n = 22$), and wild-type (WT, $n = 26$) mice are presented. (**b** and **c**) Identification of tumor cells developed in TKO mice as HS by H&E (**b**) and immunohistochemical (**c**) staining. In panel (**b**), histology of tissue sections prepared from the bone marrow (BM), spleen, and liver of TKO mice is presented. Tumor cells are diffusely distributed (top), and are large and rounded and have atypical nuclei (bottom). Arrows indicate representative tumor cells. Scale bars show 100 (top) and 50 μm (bottom). In panel (**c**), tumor cells in the BM, spleen, and liver of TKO mice are stained with antibodies to Mac-2, F4/80, and CD68, but not by antibodies to CD3 and B220. Many of the tumor cells are stained with antibodies to PCNA and Ki67. Arrows indicate representative tumor cells. Scale bars show 100 and 20 μm (inset). (**d**) Association of the early lethal phenotype of TKO mice with HS. The percentage of mice that are HS positive in the BM, spleen, and/or liver was determined for each genotype and condition (dead/moribund or not) and is presented along with the exact fraction in parenthesis. Dead/moribund TKO mice at 52–64 weeks of age showed a significantly higher rate of HS positivity than TKO mice that were neither dead nor moribund (control). No tumor was observed in the age-matched WT mice. TKO mice that died or became moribund before 52 weeks of age also showed a high rate of HS positivity. Fischer's exact test was used to calculate the statistical significance. *$P < 0.05$.

are characteristic of HS tumor cells (Figure 1b). Moreover, tumors showed monotonous morphology with tissue destruction and mass lesions, suggesting a malignant transformation (Figure 1b and Supplementary Figure S2). Immunohistochemical staining revealed the abnormal cells to be positive for the histiocyte/macrophage markers, Mac-2, F4/80, and CD68, but negative for the lymphocyte markers, CD3 and B220, consistent with the interpretation that these tumor cells represent HS (Figure 1c).[21,22] In addition, these cells stained positively with antibodies to MHC class II molecules, a marker of antigen-presenting cells including macrophages (Supplementary Figure S3). Further immunohistochemical examination showed HS cells to stain positively with antibodies to PCNA and Ki67, confirming that these cells are proliferative (Figure 1c).

As most patients with HS die of progressive disease, we examined whether HS is associated with the early lethal phenotype of TKO mice. As shown in Figure 1d, 9 of 11 moribund or dead mice at <52 weeks of age that could be necropsied bore HS in the bone marrow, spleen, and/or liver. Furthermore, no similar cells were found in WT mice even at 52–64 weeks of age. By contrast, 12 of 17 dead or moribund mice that could be necropsied at matched age developed HS, whereas only 3 of 13 nondead/moribund mice were positive for the tumor, indicating a significant link of HS with the lethal phenotype of TKO mice (Figure 1d).

HS Developed in TKO Mice is Highly Invasive and Transplantable

To evaluate the tumorigenic potential of HS developed in TKO mice, we wished to know whether the tumors spread into multiple organs. We examined six organs, the bone marrow, spleen, lung, skeletal muscle (femoral muscle), liver, and kidney. In all, 17 of the 21 tumor-bearing TKO mice at 25–66 weeks of age whose organs could be analyzed at necropsy showed multiple organ spread of the tumor (Figure 2a). As nearly all (20 of 21) TKO mice had HS in the bone marrow, this tissue might be the primary site of tumorigenesis in these mice (Figure 2b). Moreover, the majority (11 of 20) of bone marrow lesions were associated with osteolytic and direct invasions to the skeletal muscle (Figure 2b and c), indicating a strong invasiveness of the tumor. Next, to further examine the tumorigenic potential of HS developed in TKO mice, we examined whether the tumor was transplantable. Cells prepared from bone marrow or spleen of TKO mice or WT controls at 78–88 weeks of age were transplanted into lethally irradiated WT recipients intravenously. The mice transplanted with TKO cells from HS-positive tissues died within 10 weeks after transplantation, whereas control recipients that had been transplanted with cells from WT animals or from HS-negative sources of TKO animals survived, suggesting that the tumor was transplantable (Figure 3a). Indeed, histological study con-

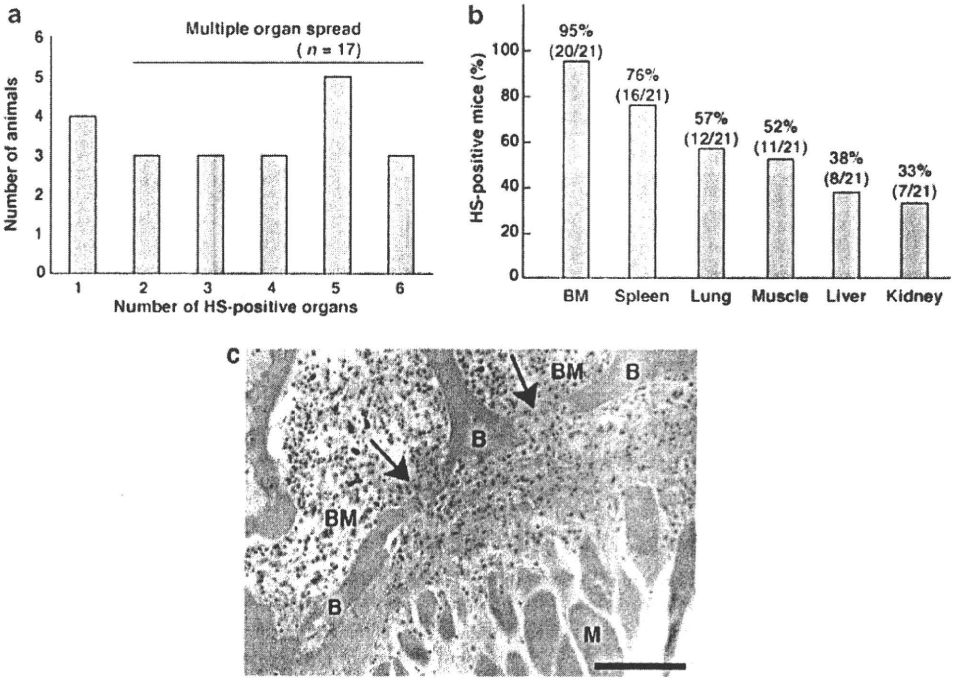

Figure 2 TKO mice show multiple organ spread of HS. (**a**) The number distribution of HS-affected organs in individual TKO mice. The bone marrow, spleen, lung, skeletal muscle, liver, and kidney from a total of 21 TKO mice were examined, and the number of HS-positive organs in each mouse was counted and plotted against the number of the corresponding mice. In all, 4 mice had only one HS-positive organ but the remaining 17 mice showed multiple organ spread of the tumor. (**b**) The organ distribution of HS positivity in TKO mice. From the data obtained in (**a**), the percentage of HS positivity by organ in affected mice is presented along with the exact fraction in parenthesis. (**c**) Histology of osteolytic invasions of HS into the skeletal muscle in TKO mice (H&E staining). B, bone; BM, bone marrow; M, muscle. Arrows indicate osteolytic lesions. Scale bar shows 100 μm.

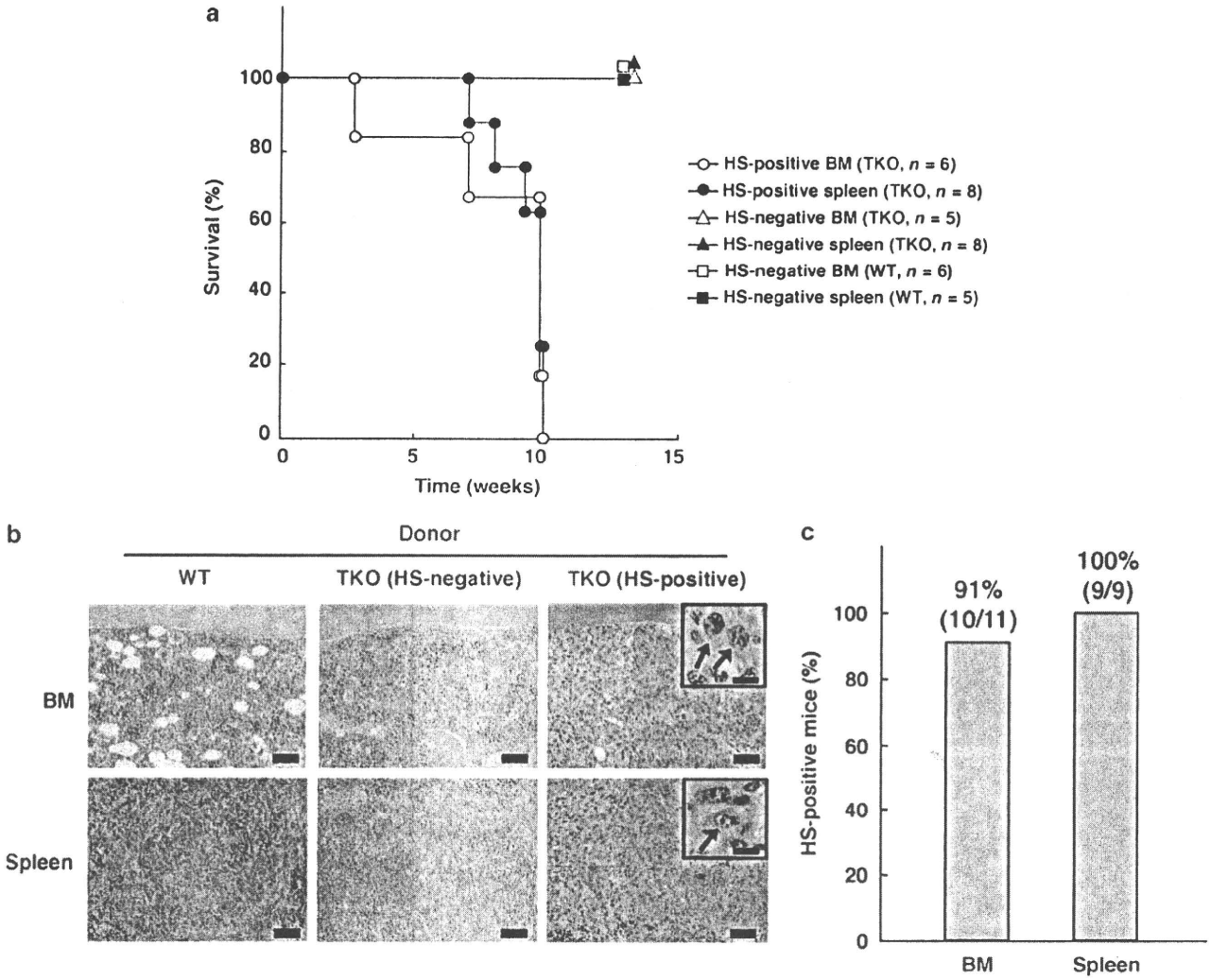

Figure 3 HS developed in TKO mice is transplantable. (**a**) Survival of transplanted mice. The survival rates of lethally irradiated (9 Gy) wild-type (WT) recipients that were transplanted with mononuclear cells from the bone marrow (BM) and spleen of the indicated donors are plotted against time after transplantation. The donor organs were scored as HS positive or negative, and only the recipients transplanted with WT cells or HS-negative TKO cells survived beyond 10 weeks after transplantation. (**b**) Histological analysis of transplanted mice. Histology of the BM and spleen sections prepared from the recipient mice that were transplanted with BM cells from the indicated donors is presented (H&E staining). Arrows indicate representative tumor cells. Scale bars show 100 and 20 μm (inset). (**c**) The percentage of HS-positive BMs and spleens in transplanted mice. The BMs and spleens from eleven and nine recipients, respectively, which had been transplanted with the HS-positive BM or spleen cells were examined. The percentage of HS positivity for BM and spleen was calculated and is presented along with the exact fraction in parenthesis.

firmed that almost all the recipient mice that had been transplanted with TKO cells from HS-positive animals and could be necropsied had HS in their bone marrow (10 of 11) and spleen (9 of 9) (Figure 3b and c). Furthermore, HS was not observed in control recipients that had been transplanted with WT cells ($n = 11$) or TKO cells from HS-negative sources ($n = 13$). We conclude that the malignant cell type that develops in TKO mice is highly invasive and transplantable.

TKO Mice Show Abnormal Accumulation of Macrophages in the Lung

During the course of histological analysis of TKO mice, we frequently found abnormal accumulation of Mac-2-positive macrophages in the lung (Figure 4a). These macrophages had a large cytoplasm without nuclear atypia and were not associated with tissue destruction, suggesting a non-tumorigenic nature. Almost all (13 of 14) TKO mice, but neither *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$ (0 of 11) mice nor the WT controls (0 of 9), exhibited such abnormal proliferation of macrophages, which was uncorrelated with the presence or absence of HS at 50–59 weeks of age (Figure 4b). The incidence of macrophage accumulation in the lung was intermediate (3 of 8) in age-matched *Dok-3*$^{-/-}$ mice. Although development of lung adenocarcinoma, but not aggressive HS, has recently been reported in mice lacking Dok-1, Dok-2, and/or Dok-3 on a pure 129S1/SvImj genetic background even at the age of 11–15 months,[23] in this study mice mu-

tated in the same genes, but on a 129/SvJ and C57BL/6 mixed background, did not exhibit elevated incidence of lung adenocarcinoma. These different findings are likely due to the difference in the genetic backgrounds.

When bone marrow cells of TKO mice at 6–10 weeks of age were transplanted into lethally irradiated recipients, we invariably observed macrophage accumulation in the lung within 10 weeks after transplantation ($n=4$), but not when bone marrow cells were transplanted from WT controls (Figure 4c). Similarly, the histological study of TKO mice at 8–12 weeks of age, before the onset of observable HS, confirmed abnormal proliferation of macrophages in the lung of all mutant mice (8 of 8) but not in WT controls (0 of 8) (Figure 4d). Again no age-matched *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$ (0 of 5) mice, and only a subset of *Dok-3*$^{-/-}$ mice (1 of 5), showed such accumulation of macrophages. Given that Dok-1 and Dok-2 negatively regulate proliferation of bone marrow-derived macrophages *in vitro*,[7] these findings suggest that Dok-1, Dok-2, and Dok-3 cooperatively inhibit the proliferation of macrophages.

Dok-1, Dok-2, and Dok-3 are Negative Regulators of Proliferative Response of Macrophages to M-CSF or GM-CSF

To address whether Dok-1, Dok-2, and Dok-3 are negative regulators of macrophage proliferation, we examined growth responses of bone marrow-derived macrophages from WT, *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$, *Dok-3*$^{-/-}$, and TKO mice at 10–12 weeks of age on stimulation with M-CSF and GM-CSF, both of which are critical for proliferation of macrophages.[24–26] TKO

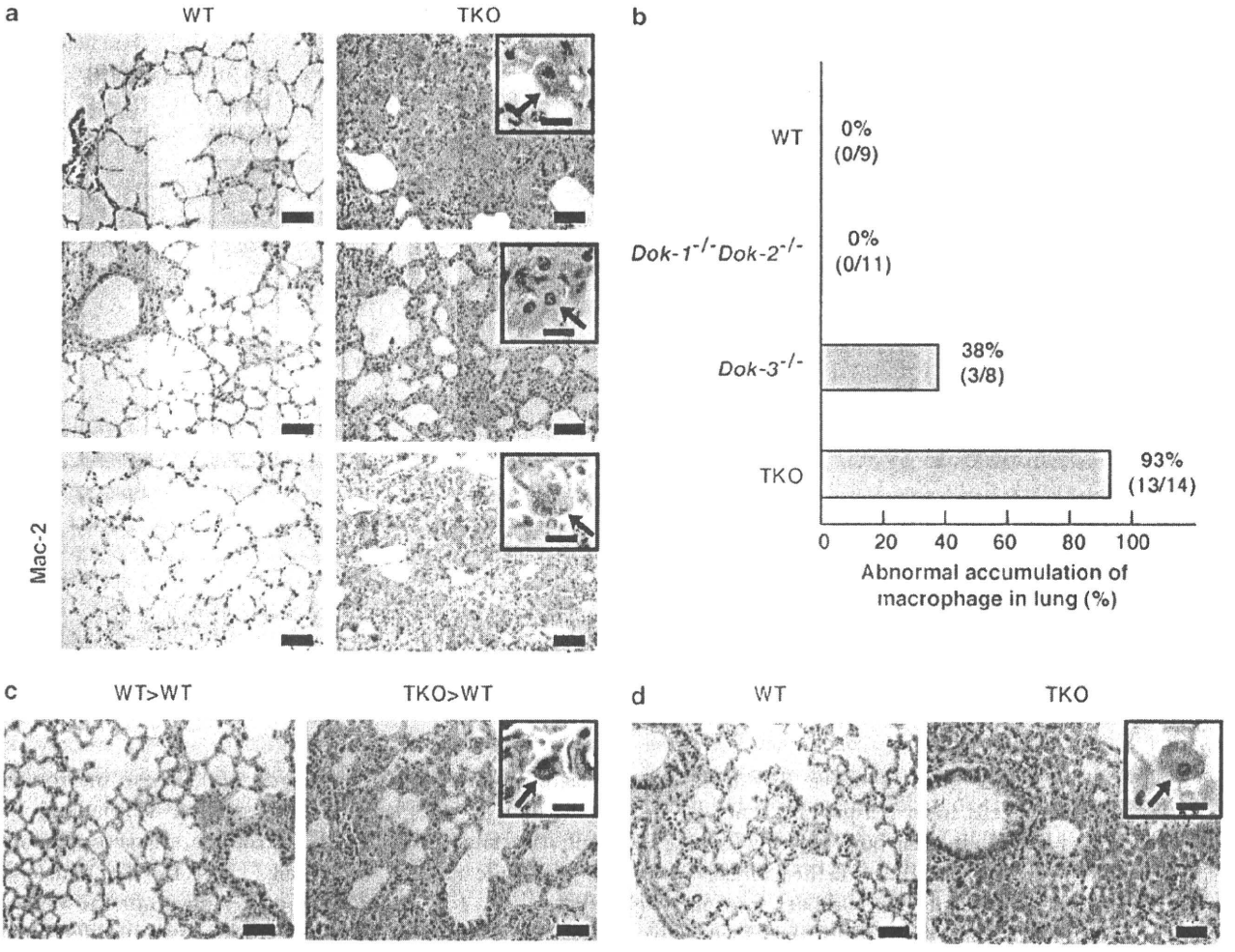

Figure 4 TKO mice show abnormal proliferation of macrophages in the lung. (**a**) Abnormal accumulation of macrophages in the lungs of TKO mice. Histology of lung sections prepared from wild-type (WT, left) and TKO (right) mice is presented (top, H&E staining; bottom, anti-Mac-2 staining). Arrows indicate representative macrophages present in the lung. Scale bars show 100 and 20 μm (inset). (**b**) The percentage of lungs with abnormal macrophage accumulations in various mice. The lungs from mice of the indicated genotypes were examined. The percentage of mice with abnormal lung macrophage accumulations was calculated and is presented along with the exact fraction in parenthesis. (**c**) Histology of lung sections prepared from recipient mice transplanted with bone marrow cells from WT (WT>WT, left) and TKO (TKO>WT, right) mice (H&E staining). An arrow indicates a representative macrophage present in the lung. Scale bars show 100 and 20 μm (inset). (**d**) Abnormal accumulation of macrophages in the lungs of younger (8–12 weeks of age) TKO mice. Histology of lung sections prepared from WT (left) and TKO (right) mice is presented (H&E staining). An arrow indicates a representative macrophage present in the lung. Scale bars show 100 and 20 μm (inset).

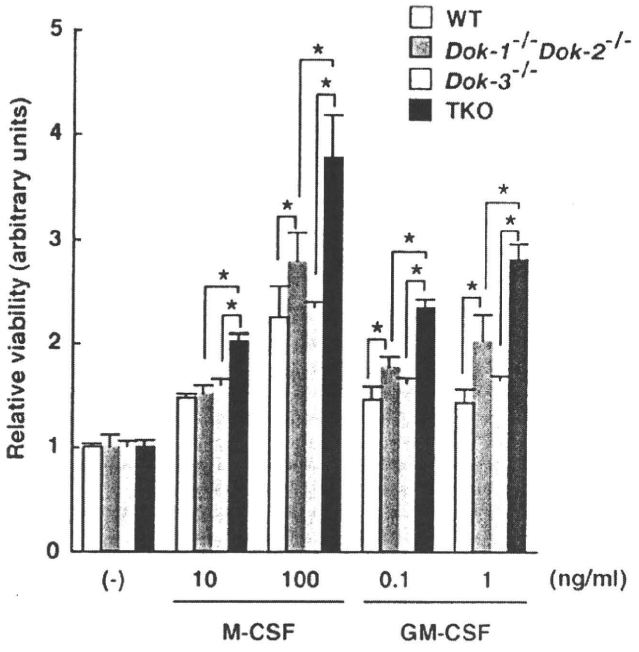

Figure 5 Augmented proliferative responses of bone marrow-derived macrophages prepared from TKO mice. Relative viabilities of bone marrow-derived macrophages of the indicated genotypes were evaluated by the MTT cell viability assay after 5 days of culture in the presence of M-CSF or GM-CSF at the indicated concentrations, in which the mean value in the absence of cytokine (−) was defined as 1 in arbitrary units for each genotype. WT, wild type. Data are expressed as mean ± s.d., and *P* values are calculated between WT and *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$ or between TKO and *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$ or *Dok-3*$^{-/-}$ macrophage proliferation. $^{*}P < 0.05$.

macrophages showed the highest proliferative response (Figure 5), consistent with the view that Dok-1, Dok-2, and Dok-3 are negative regulators of the proliferative response of macrophages to M-CSF or GM-CSF.

DISCUSSION

In this study, we have established that combined ablation of the adaptor proteins Dok-1, Dok-2, and Dok-3 has profound phenotypic consequences in mice. TKO mice, but not WT controls, develop and succumb to aggressive HS with multiple organ invasion. Although individual Dok proteins have the ability to inhibit PTK-mediated oncogenic signaling,[5,9,16] in this study mice lacking Dok-3 alone or Dok-1 and Dok-2 in combination did not develop aggressive tumors. The simplest interpretation for these data is that Dok proteins 1–3 can each suppress the aggressive transformation of HS. In addition, combined loss of Dok-1, Dok-2, and Dok-3 causes abnormal proliferation of macrophages in the lung, observable before the onset of morphologically recognizable HS. By contrast, combined ablation of Dok-1 and Dok-2 did not cause accumulation of macrophages in the lung. Although deficiency in Dok-3 caused abnormal proliferation of macrophages in the lung, the incidence was low. Therefore, the data suggest that these Dok proteins mutually compensate to inhibit the proliferation of macrophages. Indeed, our *in vitro* assay revealed that these proteins cooperatively downregulate proliferative response of macrophages on M-CSF or GM-CSF stimulation. Given that histiocytes are tissue-resident macrophages and HS is believed to arise from macrophage lineages, it is likely that the enhanced proliferative capacity of macrophages in mice lacking Dok-1, Dok-2, and Dok-3 contributes to the development of HS.

In general, Dok family proteins are believed to be activated as adaptors by tyrosine phosphorylation.[9] It was previously demonstrated that Lyn is required for the tyrosine phosphorylation of Dok-1 and Dok-3 in B cells (in which Dok-2 is typically undetectable) on B-cell receptor signaling,[6,27] suggesting that Lyn may activate these Dok proteins as negative regulators to suppress HS. Indeed, it has been reported that Lyn is expressed in macrophages, and that bone marrow-derived macrophages from Lyn-deficient mice showed enhanced growth responses to M-CSF and GM-CSF.[28] Furthermore, mice lacking Lyn develop macrophage tumors, which may be related to HS.[28] Unlike TKO mice, however, Lyn-deficient mice additionally develop severe renal disease.[29]

As mentioned above, HS in humans is an aggressive malignancy of unknown etiology and there remains a need for realistic animal models. Mouse models that have been reported for HS frequently show multiple lesions including lymphomas and severe renal failure. For example, the majority of mice lacking Pten, Ink4A, and Arf in combination develop both lymphoblastic B-cell lymphomas and HS with virtually the same incidence.[30] Similarly, mice lacking the cell-cycle regulator p21 develop a variety of tumors, including HS and B-cell lymphomas, and also suffered from severe renal failure,[31] unlike TKO mice. The syndrome elicited in mice lacking all the three proteins, Dok-1, Dok-2, and Dok-3, more specifically resembles the disease found in humans and hence may serve as a useful model for the study of HS. Although elucidation of the mechanisms by which the ablation of Dok proteins specifically causes HS and how the tumor gains its aggressive phenotype awaits further studies, such studies will help unveil the hidden etiology of this rare aggressive human malignancy.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Utility and Limitations of SP600125, an Inhibitor of Stress-Responsive c-Jun N-Terminal Kinase

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Abstract: Stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) belongs to the mitogen-activated protein kinase (MAPK) family and plays an important role in many biological contexts. JNK is deeply involved in several serious human disorders, including inflammation, obesity, diabetes, neuronal disease and cancer. Accordingly, JNK has been recognized as an appropriate target for the treatment of these diseases, and much effort has been expended over the past 15 years to isolate JNK inhibitors that can inactivate this kinase. In 2001, the compound SP600125 was reported as the first JNK-specific inhibitor. Many researchers have subsequently employed SP600125 in *in vitro* and *in vivo* models to evaluate whether certain disease-associated events are JNK-dependent. Indeed, more than 1300 studies citing the use of SP600125 as a JNK inhibitor in cell cultures and animal models have been reported. However, although SP600125 has been employed successfully to inhibit JNK in several situations, there have been questions about its specificity for JNK. SP600125 can bind to a broad range of protein kinases and inhibit some of them with similar or greater potency than JNK, confirming that many additional kinases may be targets of SP600125. In this article, we review both the usefulness of SP60125 as a JNK inhibitor and the limitations to its specificity.

Keywords: JNK, MAP kinase, SP600125, kinase inhibitor, mast cell, IgE, PI3K, allergy.

I. INTRODUCTION

The mitogen-activated protein kinases (MAPKs) are a family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli [1-7]. Upon stimulation, MAPKs phosphorylate their specific substrates at serine and/or threonine residues. Such phosphorylation events can either positively or negatively regulate the substrate's activity, and thus the activity of the entire signaling cascade. There are three "conventional" MAPK family members: extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38-MAPK. Each of these encompasses its own subfamily: ERKs (ERK1 and ERK2), JNKs (JNK1, JNK2, and JNK3), and p38-MAPKs (p38-MAPKα, p38-MAPKβ, p38-MAPKγ, and p38-MAPKδ) [1, 3-6, 8] (Fig. 1).

Members of the JNK subfamily are activated both by cellular stresses such as UV irradiation, heat shock, cisplatinum, etoposide, thapsigargin and tunicamycin, and by inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα). Activation of a JNK enzyme requires phosphorylation of the Tyr and Thr residues located in the Thr-Pro-Tyr motif of the MAPK domain. Two kinases, MKK4 (also known as SEK1) and MKK7, are responsible for this phosphorylation [4, 5, 9]. Once activated, JNK phosphorylates a number of substrates, including the c-Jun component of the activator protein-1 (AP-1) transcription factor that regulates the expression of genes involved in stress responses.

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II. STUDIES OF JNK USING GENE DISRUPTION IN MICE

Many approaches have been taken to study JNK function, including gene targeting in mice. This work has yielded much information on JNK's roles in embryogenesis, the immune system, the neuronal system, and metabolism. In the following subsections, we summarize JNK functions revealed through the examination of mice in which the JNK gene has been disrupted. The implication of the studies outlined below is that regulation of JNK activity may be an effective means of ameliorating certain immunologic, neuronal and metabolic disorders.

i. Physiological Role of JNK in Murine Embryogenesis

Despite the importance of JNK functions to diverse essential cellular processes, single mutant mice deficient for JNK1, JNK2 or JNK3 all survive and appear morphologically normal. These data indicate that JNK1, 2, and 3 likely have redundant or overlapping functions during embryonic development. Moreover, JNK1/JNK3 and JNK2/JNK3 double mutants are also viable. In contrast, JNK1/JNK2 double mutants show severe dysregulation of apoptosis during brain development that result in embryonic lethality. Thus, JNK1 and JNK2 play a redundant but critical role in the regulation of region-specific apoptosis during early brain development [10].

ii. Physiological Role of JNK in the Murine Neuronal System

In situ hybridization and Northern blot studies have shown that the *jnk1* and *jnk2* genes encoding JNK1 and JNK2 are expressed ubiquitously. In contrast, the *jnk3* gene

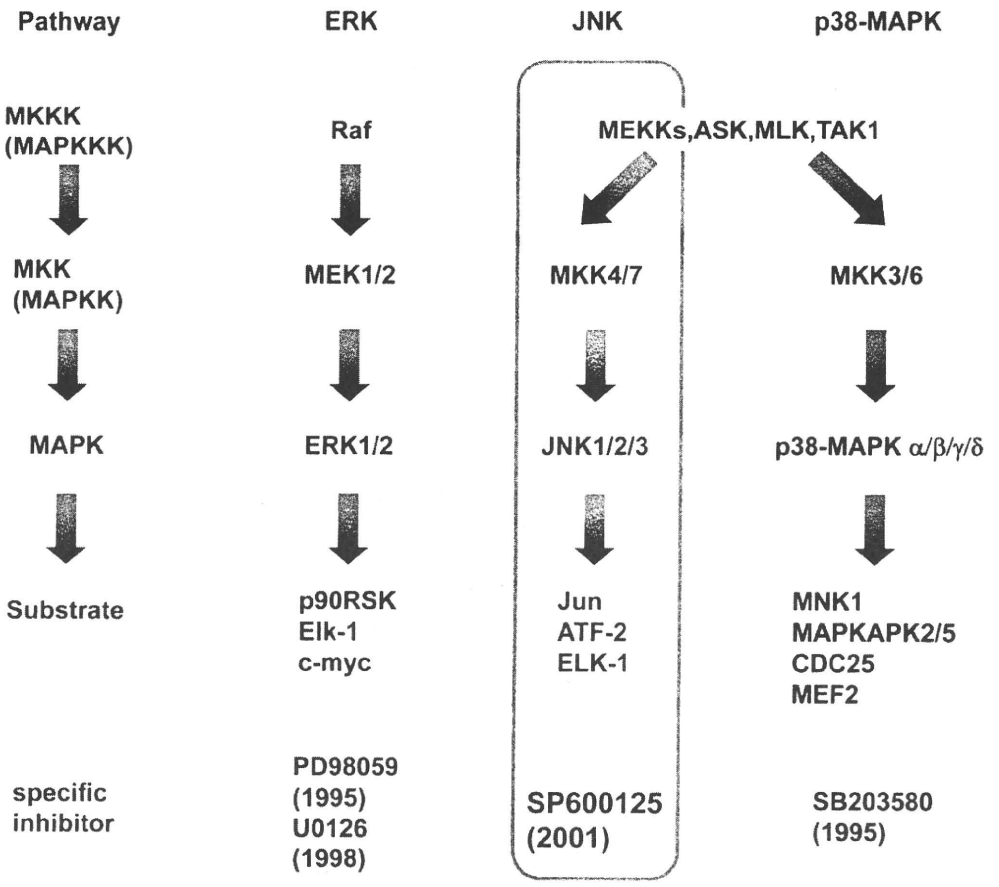

Fig. (1). The MAPK family of signaling pathways.

There are three major groups of MAPKs: ERK, JNK and p38-MAPK. Each of these is the central kinase in a pathway (left) characterized by an MAPK kinase-kinase (MKKK) and a MAPK kinase (MKK) that respond to different stimuli to activate the MAPK enzyme (ERK, JNK or p38-MAPK) and allow it to act on its substrate. Specific inhibitors (with their dates of discovery) are shown for each pathway.

has a more limited pattern of expression and is largely restricted to brain. Mice deficient in JNK3, or mice with mutations in the phosphorylation site of c-Jun, are resistant to the hippocampal neurotoxic events associated with administration of the glutamate receptor agonist kainic acid [11]. These results suggest that JNK3 is involved in glutamate excitotoxicity, an important component of ischemic neuronal organismal death. Targeted deletion of JNK3 has been shown to protect mice from brain injury after cerebral ischemia-hypoxia [12], leading to the proposal that JNK3 may be a potential target for neuroprotection therapy in stroke patients [9].

iii. Physiological Roles of JNK in the Murine Immune System

Although mice deficient for JNK1 or JNK2 appear to be morphologically normal, these mutants are immunodeficient due to severe defects in T cell function. JNK signaling activates the IL-2 promoter and thus plays an important role in T cell activation. In particular, mice lacking JNK1 or JNK2 exhibit deficits in $CD4^+$ T-helper cell function. In culture, JNK1-deficient $CD4^+$ T cells selectively differentiate into Th2 cells [13], and JNK2-deficient $CD4^+$ T cells also fail to differentiate into Th1 cells [14]. Intriguingly, JNK1 and JNK2 have differing roles in $CD8^+$ T cells [15, 16]. JNK1-deficient mice exhibit defective $CD8^+$ T cell expansion *in vitro* and *in vivo* and JNK1-deficient $CD8^+$ T cells show reduced expression of IL-2 and IFN-γ. In contrast, JNK2-deficient $CD8^+$ T cells express greatly increased amounts of IL-2 and IFN-γ. These data imply that JNKs play multiple roles in T cell-mediated immune responses, and that JNKs might be useful therapeutic targets. It has been suggested that controlled JNK inactivation might allow the selective modulation of effector T cell functions in diseases such as rheumatoid arthritis, asthma and chronic transplant rejection [9].

iv. Physiological Roles of JNK in Metabolism

Biochemical studies have established that JNK phosphorylates the insulin receptor substrate-1 (IRS-1) protein at its inhibitory site Ser-307 [17, 18]. JNK activation can therefore suppress signal transduction by the insulin receptor. These observations implicate the JNK signaling pathway in insulin resistance, metabolic syndrome, and type 2 diabetes. Indeed, ablation of the JNK pathway in mice can influence their susceptibility to obesity and diabetes [19] . For exam-

ple, knockout mice that lack expression of JNK1, or the JNK scaffolding protein known as JNK-interacting protein-1 (JIP1), are resistant to the effects of a high-fat diet and do not develop obesity or insulin resistance [20-22].

III. SP600125 AS A JNK INHIBITOR

Inhibitor studies are another important means by which enzyme functions can be revealed. The first inhibitors of MAPK activity targeted ERK and p38 MAPK were reported in 1995 (Fig. **1**), and many studies centered on the use of these inhibitors have been reported since then. In contrast, an inhibitor of JNK activity was developed only in 2001 [23]. Anthrax [1,9-cd] pyrazol-6 (2H)-one, known less formally as SP600125, was the first compound used to study the function of JNK both in cells and in whole animals. JNK inhibition by SP600125 was observed to be reversible and ATP-competitive, and showed IC_{50} values for JNK inhibition in the range of 40–90 nM. In addition, SP600125 exhibited >300-fold selectivity for JNK over ERK1 and p38-MAPK, and 10-100 fold selectivity for JNK over 14 other protein kinases. These results suggested that SP600125 bound specifically and with high affinity to residues in JNK's ATP-binding site [23].

At the level of cellular functions, SP600125 treatment prevents the expression of several anti-inflammatory genes in cell-based assays [23], the activation of AP-1 and expression of collagenase-3 in synoviocytes [24], the expression of type IV collagenase in ovarian cancer (OVCAR) cells [25], and the activation and differentiation of primary human $CD4^+$ cell cultures [23]. In animal studies, SP600125 blocks lipopolysaccharide-induced expression of TNFα and also inhibits anti-CD3-induced apoptosis of $CD4^+$ and $CD8^+$ thymocytes [23].

Since the initial studies of SP600125 conducted in 2001-2002, this inhibitor has become increasingly popular for two reasons. First, prior to the advent of SP600125, it was possible to study the contribution of JNK signaling to biological processes only *via* the introduction of active or dominant-negative JNK pathway components into cells, most often by transfection. Although much valuable information on the roles of JNK pathways in mammalian cells has been garnered from this method, the process of transfection itself alters the cell for many hours or days prior to the experiment of interest. This delay is a drawback that does not exist with chemical inhibitors, which can be rapidly applied to cells either *in vitro* or *in vivo*. For example, the inhibitors PD98059 and U0126 target the MEK1 and MEK2 kinases that act directly upstream of ERK [26]. Similarly, the inhibitor SB203580 and related pyridinylimidazole compounds directly inhibit p38 MAPK (reviewed in Refs. [27, 28]). The use of this panel of inhibitors has greatly accelerated the understanding of MAPK pathways. The second, more mundane reason for SP600125's popularity is its commercial availability. Other compounds including CEP-1347 have been reported [29, 30] but has yet to be commercially released [31].

More than 1300 reports citing the use of SP600125 as a JNK inhibitor in cell cultures and animal models have been published. We have outlined a number of representative *in vitro* and *in vivo* studies below and summarized them in Table **1**. The use of SP600125 has revealed much new knowledge about JNK functions that was not exposed by gene-disruption or over-expression studies. However, this work has also made it clear that, because of specificity concerns, SP600125 should be used only to demonstrate JNK involvement in a process. This issue is addressed in detail in section IV.

i. SP600125 and Arthritis

Several studies have reported the effects of the JNK inhibitor administration in animal models of arthritis. SP600125 blocks IL-1-induced phosphorylation of JNK and c-Jun in cultured synoviocytes from rheumatoid arthritis patients, and impairs the production of matrix metalloproteinase-13 (MMP-13), an enzyme associated with cartilage destruction [24]. SP600125 administration also inhibits JNK activation and collagenase expression in the joints of rats with adjuvant arthritis. These animals showed significant reductions in paw swelling and damage to bone and cartilage [24]. These studies were extended by using JNK2 knockout mice in a model of passive murine collagen-induced arthritis. This work showed that JNK2 is a key determinant of matrix degradation, but is less important for inflammation and paw swelling [32]. In light of these findings, Manning and Davis have proposed that the inhibition of JNK should be considered as a potential therapy for rheumatoid arthritis [9].

ii. SP600125 and Abdominal Aortic Aneurysm

In humans, examination of tissue samples from abdominal aortic aneurysms has shown the presence of upregulated MMP-9 and activated JNK [33]. Treatment of these samples *ex vivo* with SP600125 repressed the release into the culture supernatants of MMP-9, implicating JNK in tissue damage associated with abdominal aortic aneurysms. This hypothesis has also been confirmed by the work of Yoshimura *et al.*, who have used SP600125 and mouse model to show that JNK is critical for the development of abdominal aortic aneurysms. Treatment with SP600125 prevented the development of abdominal aortic aneurysms in $CaCl_2$-induced mouse model. In particular, SP600125 treatment almost completely prevented aortic dilatation and medial thinning while preserving the integrity of the elastic lamellae [33].

iii. SP600125 and Ischemia

Okuno *et al.* showed that JNK inhibition by SP600125 was potently effective in decreasing neuronal apoptosis in the ischemic core after *in vivo* transient focal cerebral ischemia (tFCI) [34]. During ischemia, BimL (Bim long) is induced and phosphorylated in parallel with induction of JNK activity. Co-immunoprecipitation studies have consistently revealed increased interaction of JNK with BimL, as well as BimL with the cell death effector Bax, after tFCI. SP600125 blocked these interactions at a dose that significantly inhibited JNK-induced neuronal apoptosis. That neurons were protected from ischemia-induced apoptosis was determined by TUNEL staining and an apoptosis-related DNA fragmentation assay. Biochemically, SP600125 blocked translocation

Table 1. JNK Functions Revealed by the Use of SP600125 in Mammalian Cells and Animal Models. Representative Studies are Cited

In Vitro Study		
Cell Type	**Function**	**Refs.**
rat cardiomyocyte cell line H9c2	apoptosis	Uetani *et al. J. Biol. Chem.* **2009**
rat neonatal cardiomyocytes	apoptosis	Xie *et al. J. Biol. Chem.* **2009**
human EBV-transfomed B cells	apoptosis	Kim *et al. J. Immunol.* **2008**
human umbilical vein endothelial cells	apoptosis	Ho *et al. J. Biol. Chem.* **2008**
human cholangiocarcinoma cell line KMCH expressing Mcl-1 siRNA	apoptosis	Werneburg *et al. J. Biol. Chem.* **2007**
multidrug-resistant MCF7/ adriamycin-resistant (ADR) human breast carcinoma cells	apoptosis	Lee *et al. J. Biol. Chem.* **2007**
rat primary microglia	apoptosis	Yang *et al. J. Immunol.* **2006**
human bladder cancer cell line RT112, 253J, J82 human prostate cancer cell line LNCap	apoptosis	watanabe *et al. Oncogene* **2006**
rat neural stem cells	apoptosis	kanzawa *et al. Oncogene* **2006**
mouse primary cultures of striatal neurons	apoptosis	Charvin *et al. Proc. Natl. Acad. Sci. U S A* **2005**
human foreskin-derived keratinocyte	gene expression	Wehkamp *et al. J. Invest. Dermatol.* **2006**
human hepatocellular carcinoma cell line HuH-7	gene expression	Higuchi *et al. J. Biol. Chem.* **2004**
primary cultures of human hepatocytes	gene expression	Holt *et al. Genes. Dev.* **2003**
mouse primary splenocytes	gene expression	Brint *et al. J. Biol. Chem.* **2002**
human lung epithelial fibroblast cell line WI38	autophagy	Oh *et al. J. Pharmacol. Exp. Ther.* **2009**
mouse NF-1(+/-) microglia	proliferation, motility, tumor	Daginakatte *et al. Cancer Res.* **2008**
mouse sertori cell line TM4 cells	mRNA degradation	Sze *et al. Biochem. J.* **2008**
human hepatoma cell line Hep3B	inflammation	Nishikawa *et al. J. Immunol.* **2008**
rat fibloblast SCN, pineal gland lung explants	circadian rhythms	Chansard *et al. Neuroscience* **2007**
humam epidermal keratinocytes	differentiation	Gazel *et al. J. Biol. Chem.* **2006**
In Vivo Study		
Model Type	**Function**	**Refs.**
mouse corneal neovascularization model	apoptosis	Ho *et al. J. Biol. Chem.* **2008**
mouse acetaminophen-induced liver injury model	liver injury	Hanawa *et al. J. Biol. Chem.* **2008**
mouse kappa opioid receptor-mediated analgestic model	analgestic response	Bruchas *et al. J. Biol. Chem.* **2007**
mouse Group B streptococcus induced septic shock model	sepsis	kenzel *et al. J. Immunol.* **2006**
mouse LPS-induced lung inflammation model	lung inflammation	Arndt *et al. J. Immunol.* **2005**
mouse antigen-induced airway inflammation model	asthma	Nath *et al. Eur. J. Pharmacol.* **2005**
mouse abdominal aortic aneurysm model	Abdominal aortic aneurysm	Yoshimura *et al. Nat. Med.* **2005**
rat focal cerebral ischemia model	ischemia	Okuno *et al. J. Neurosci.* **2004**
rat adjuvanti-induced arthritis model	inflammatory arthriris	Han *et al. J. Clin. Invest.* **2001**

of Bax from the cytosol to the mitochondria after tFCI. These results suggest that the JNK signaling pathway is involved in ischemia-induced neuronal apoptosis because it stimulates Bax translocation to the mitochondria. In this context, BimL is likely regulated by JNK as a downstream substrate for the transmission of apoptotic signals to Bax. Thus, this use of SP600125 has provided evidence for a role of the JNK signaling pathway in ischemia-induced neuronal apoptosis.

iv. SP600125 and Asthma

The lung inflammation associated with asthma is induced by cytokine and chemical mediators produced by activated mast cells and basophils. These cell types express high levels of surface IgE receptors (FcεRI) that can bind to a range of IgE antibodies. When these IgE antibodies are engaged by multivalent antigen, the FcεRI molecules are crosslinked and initiate intracellular signaling that leads to cellular activation

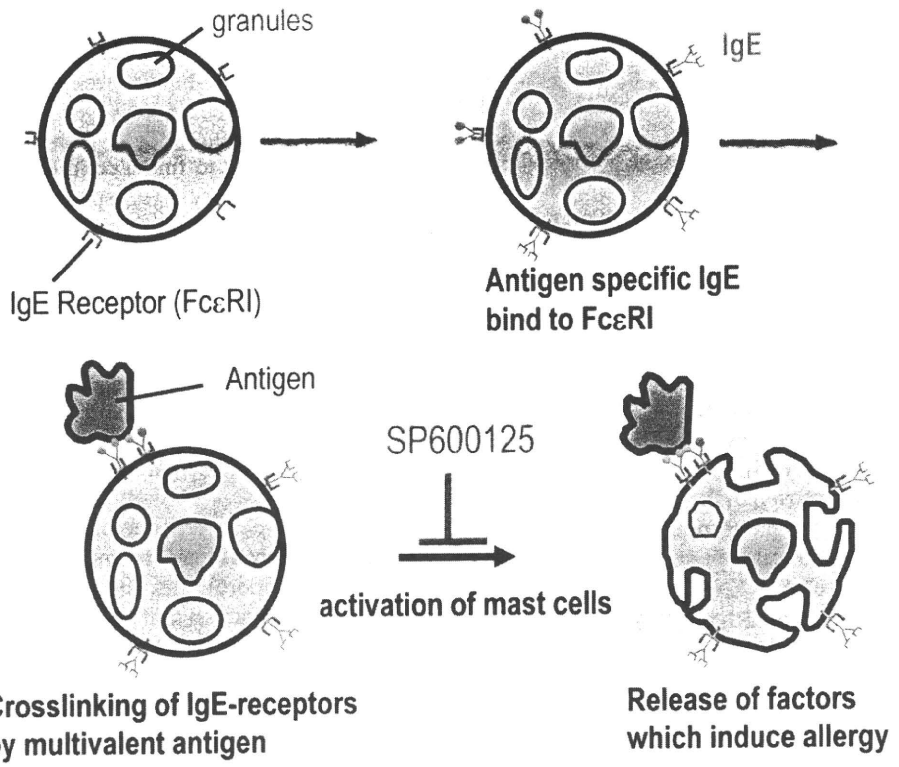

Fig. (2). Triggering of mast cell degranulation.

Mast cells express high levels of FcεRI molecules that can bind to a wide variety of IgE antibodies. The binding of specific multivalent antigen to certain of these fixed IgE antibodies crosslinks the FcεRI molecules and triggers mast cell activation. Activated mast cells release biogenic amines such as histamine and serotonin from their granules, as well as inflammatory cytokines (TNFα, IL-6, IL-13 and others) and lipid mediators (PGD2, LTB4 and others). These factors induce the early-phase allergic reaction and contribute to the inflammatory cell infiltration characteristic of asthma.

(Fig. 2). Areas of asthmatic inflammation are also characterized by an accumulation of eosinophils, which play an important role in the exacerbation of asthma [35].

Nath *et al.* have used SP600125 to evaluate the function of JNK in a murine model of allergic asthma [36]. Sensitized Balb/C mice subjected to chronic allergen exposure show an accumulation of inflammatory cells, increased numbers of airway smooth muscle cells and goblet cells, and heightened bronchial responsiveness. Pretreatment with SP600125 attenuated allergen-induced bronchial hyperresponsiveness and significantly inhibited eosinophil and lymphocyte accumulation in bronchoalveolar lavage fluid. This amelioration coincided with a decrease in the peroxidase-positive eosinophils within the bronchial submucosa. SP600125 also inhibited allergen-induced increases in airway goblet cells and smooth muscle cells. These observations indicate that JNK activity may be important for the chronic inflammation, airway remodelling and bronchial hyperresponsiveness induced by chronic allergen exposure in the mouse. A decrease in the concentrations of IL-4, IL-13, RANTES (Regulated on Activation, Normal T Expressed and Secreted) and TNFα in the lung was also observed in this model following SP600125 administration [36]. These results suggest that SP600125 might be useful for reducing asthma-associated inflammation, particularly that dominated by mast cells, basophils, Th2 cells and eosinophils.

Taken together, all these reports indicate that SP600125 is a very promising drug with potential therapeutic applicability to a broad range of diseases. Future studies of this sort should add to the list of disorders for which SP600125 treatment may be beneficial.

IV. SPECIFICITY OF SP600125

Despite the favorable data presented above, recent work by Bain *et al.* has raised concerns about SP600125's specificity as an inhibitor of JNK [37, 38]. Fresh examination of IC_{50} values has shown that SP600125 is actually a rather weak inhibitor of JNK isoforms, and that the IC_{50} values reported previously in [23] are likely explained by the lower ATP concentrations used in these assays. The original studies of SP600125 selectivity showed little or no inhibition of the 17 protein kinases and 18 inflammatory enzymes tested [23, 24]. However, Bain *et al.* found that SP600125 was nonspecific and inhibited 13 of the 28 protein kinases tested with similar or greater potency than JNK [37]. Notably, serum and glucocorticoid-regulated kinase, p70 ribosomal S6 kinase, AMP-dependent protein kinase, cyclin-dependent kinase 3, casein kinase 1δ and dual-specificity tyrosine–regulated kinase 1A were all inhibited by 10 μM SP600125 to a greater extent than was JNK.

Fabian *et al.* have also re-examined the specificity of SP600125 [39]. These authors developed a new, more sensitive and versatile method for determining kinase inhibitor specificity based on ATP-site-dependent competition binding assays and a T7 phage that serves as a "tag" for kinases. Data obtained using this method showed that SP600125 can bind to a broad range of protein kinases (39 of the 119 tested), confirming that many additional kinases may be targets of SP600125.

The specificity of SP600125 has also arisen as an issue in our work. Our objective has been to examine the physiological role of JNK in mast cells, which play a central role in inflammatory and immediate allergic responses. Engagement of FcεRI of mast cells induces their degranulation and triggers the expression of the genes encoding the inflammatory cytokines IL-6, TNFα and IL-13 (Fig. **3**). We found that the use of SP600125 could almost completely inhibit FcεRI-induced degranulation and cytokine production by mast cells [40]. However, examination of antigen-stimulated mast cells revealed that the time course of JNK activation in these cells did not correlate with that of FcεRI-induced degranulation. Furthermore, degranulation and cytokine gene expression induced by FcεRI engagement were not impaired in MKK7-deficient mast cells, which cannot activate JNK. These results suggested that the altered phenotypes observed in our SP600125-treated mast cells were not due to effects on the JNK signaling pathway. We eventually determined that, rather than inhibiting JNK, SP600125 markedly inhibits the FcεRI-induced activation of phosphatidylinositol 3-kinase (PI3K) pathway in mast cells (Fig. **3**). PI3K is known to be important for both degranulation and cytokine gene expression in mast cells [41]. Our results indicate that SP600125 has non-JNK targets in mast cells, and that these targets may be important for antigen-induced mast cell activation. Cytokines, proteases, biogenic amines and lipid mediators secreted by mast cells induce basophils and eosinophils to congregate in inflamed tissues, so that FcεRI-activated mast cells can be said to make a major contribution to leukocyte infiltration. Therefore, the effects of SP600125 in animal models of allergy (as described above) may be explained in part by SP600125-mediated impairment of PI3K signaling in mast cells. In any case, our work implies that SP600125 may be a potent new drug for the treatment of allergy.

V. FUTURE PERSPECTIVE ON THE NOVEL STRATEGY TO DEVELOP MORE SPECIFIC INHIBITOR FOR JNK

SP600125 has been extensively used as a purported JNK inhibitor both to study the role of JNK in a cell physiology context, and to evaluate the usefulness of JNK inhibition in a therapeutic context. Indeed, SP600125 has shown striking efficacy in some disease models. Ordinarily, such results would mean that JNK might become an attractive new mechanistic target, and that SP600125 would be an effective new drug to treat human disease. However, we and others have raised concerns about the specificity of SP600125 as a JNK inhibitor. The efficacy of SP600125 in some disease models may be derived from its inhibitory effects on targets other than JNK.

Kinase inhibitors are currently under intensive study as a promising new class of targeting drugs. Most of these inhibitors target the ATP-binding region of a kinase protein, setting up a competitive binding situation. However, the structure of the ATP-binding site is very similar among kinases, making it difficult to find a competitive inhibitor of this type that has an inhibitory effect specific to the kinase of interest. Moreover, one of the reasons that kinase inhibitor treatments often generate side effects is that the inhibitor non-specifically shuts down a variety of kinases. At the time of writing, there are eight kinase inhibitor drugs on the market that are approved for use only in oncology. The treatment of chronic diseases with kinase inhibitors may require that these agents show greater selectivity for the target kinase. Kinase inhibitors that attack an allosteric domain rather than the ATP-binding site of a kinase of interest may be the answer to improving kinase specificity, since allosteric inhibitors typically alter kinase conformation and prevent protein substrate binding. Allosteric inhibitors thus may provide exciting therapeutic opportunities based on the exploitation of new mechanisms of action that decrease off-target side effects.

A number of pharmaceutical companies are using *in vitro* and *in vivo* studies to explore the therapeutic potential of JNK inhibitors. Some such inhibitors, including Celgene developing compound CC-401, are already being tested in clinical trials [42]. Other JNK inhibitors, such as CEP-1347 and the small peptide inhibitor I-JIP (Inhibitor of JIP-1), are currently more useful for studying JNK functions than for therapy. CEP-1347 inhibits members of the mixed lineage kinase (MLK) family, with IC50 values of 23-51 nM for MLL1, 2 and 3. MLK1, 2 and 3 are upstream activators of the JNK pathway [29, 30]. Thus, CEP-1347 provides a means of targeting the JNK pathway *via* inhibition of select upstream MEKKs. In contrast, I-JIP is a 21-amino acid peptide inhibitor that directly targets activated JNK enzymes. The sequence of I-JIP was derived from amino acids 143-163 of the JNK-binding domain (JBD) of JIP-1. *In vitro*, I-JIP inhibited JNK-mediated phosphorylation of recombinant c-Jun, Elk and ATF2 by up to 90% [43].

One of the most specific ways to inhibit JNKs may be to block their expression through use of antisense techniques or RNA-mediated interference approaches. This mode of inhibition has already accelerated the discovery of cellular events mediated by JNKs. Whether these types of approaches can be used therapeutically are matters for future exploration.

VI. CONCLUDING REMARKS

We believe that SP600125 can be truly effective as a therapeutic, as has been demonstrated in various animal disease models. However, this efficacy may be attributable to SP600125's inhibition of kinases other than JNK. Future studies employing SP600125 should endeavor to ascertain its true target, so that the production of an inhibitor specific for the kinase responsible for the observed effect can be made. Such an approach should lead to the development of medicines that have greater drug efficacy and fewer adverse effects.

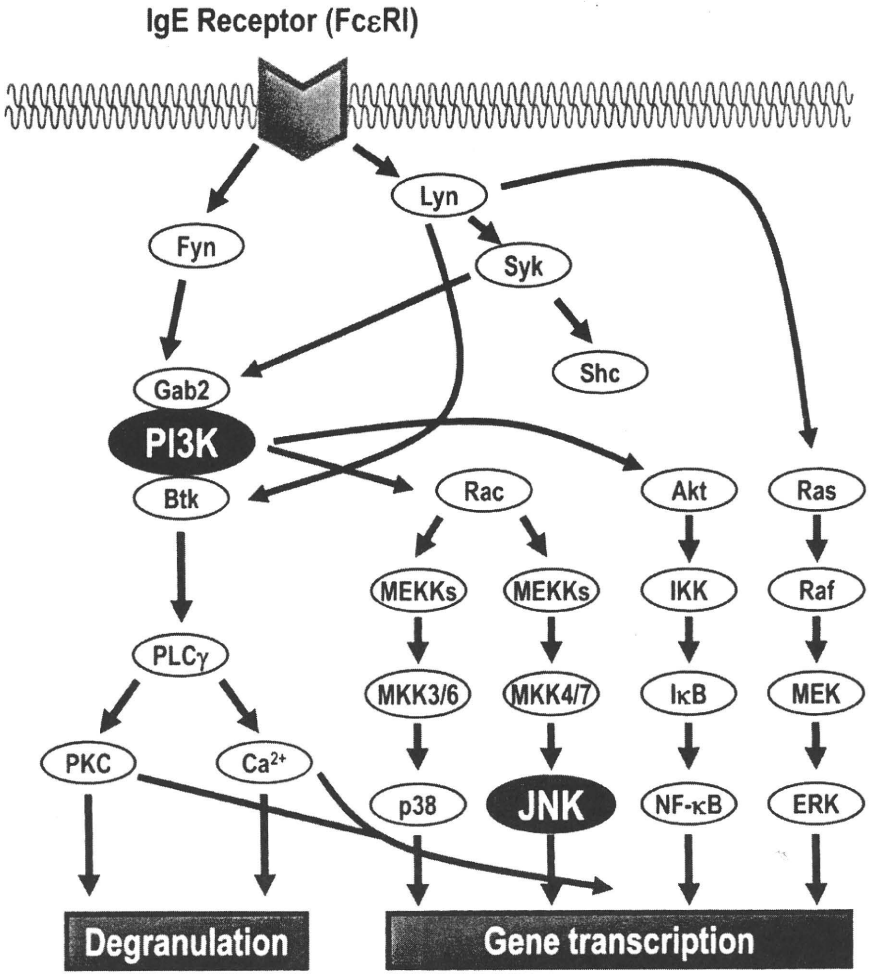

Fig. (3). Signal transduction pathways triggered by FcεRI aggregation.

Aggregation of FcεRI molecules on a mast cell triggers the activation of protein tyrosine kinases (PTKs) such as Syk, Fyn and Lyn. Activated Fyn acts on the scaffolding adaptor protein Grb-2 associated binder (Gab2) to relay the signal from FcεRI to PI3K. PI3K then triggers downstream activation of PKC and calcium signaling, which are essential for mast cell degranulation. Aggregation of the FcεRI also activates MAPKs, particularly ERK, JNK and p38 MAPK. Activation of these kinases is important for the transcription of cytokine genes. Activation of PI3K leads to stimulation of the Akt pathway, which also triggers gene transcription. SP600125 inhibits both PI3K and JNK in mast cells.

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Short Communication

Expression of the RNA-binding protein Musashi1 in adult liver stem-like cells

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Aim: Musashi1 is an RNA-binding protein that regulates the Notch signaling pathway in stem cells. Our previous study revealed that Musashi1 is expressed in early hepatocytes during liver development in the mouse. However, whether this unique protein is expressed with Notch signaling markers in adult liver stem-like cells remains unknown.

Methods: Established hepatic stem-like cells (HSLC), which were derived from adult Sprague–Dawley rats, were used for experiments *in vitro*. HSLC were differentiated into mature cells in terms of producing albumin when co-cultured with epidermal growth factor (EGF). The mRNA expression of *Musashi1*, *Notch* family (*Notch1* and *Notch2*), *Jagged1* and *Hes1* was examined in HSLC before and after cell differentiation using polymerase chain reaction-based techniques. Protein expression of Musashi1 was examined in the HSLC and normal mature hepatocytes by immunofluorescence staining.

Results: The mRNA expression of *Musashi1*, *Notch1*, *Jagged1* and *Hes1* was detected in the original HSLC before culturing with EGF but not in primary cultured mature hepatocytes. The mRNA expression of *Musashi1* and *Hes1* was found to be downregulated in differentiated HSLC that produce albumin. Protein expression of Musashi1 was detectable in the original HSLC but not in both differentiated HSLC and mature hepatocytes.

Conclusion: These findings demonstrate that the RNA-binding protein Musashi1 is expressed with Notch signaling markers in adult liver stem-like cells.

Key words: hepatic stem cell, Musashi1, Notch, liver, RNA-binding protein

INTRODUCTION

IT HAS BEEN demonstrated that liver cell regeneration originates from epithelial cells through two mechanisms. First, mature hepatocytes can proliferate independently by division after the loss of liver cells, as is often observed after a partial hepatectomy.[1] An alternative mechanism, in which liver stem/progenitor cells that subsequently differentiate into hepatocytes, cholangiocytes or other liver components are produced, is involved in reconstruction of the liver after severe liver damage.[2] The signal transduction in liver stem cell differentiation has not been fully investigated.

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Musashi1, a neural RNA-binding protein was first isolated as a mammalian homolog of the *Drosophila* protein, which is required for the asymmetric division of sensory neural precursor cells.[3] It is also known that Musashi1 is a positive regulator of the Notch signaling pathway,[4,5] which is essential for the determination of cell fate,[6] thereby maintaining the self-renewing ability of stem cells. Thus, Musashi1 is closely involved in the regulation of asymmetric cell division of stem-like cells, which generates differentiated cells.

In our previous study, we have shown that Musashi1 is expressed in early hepatocytes during liver development in the mouse.[7] Whether this unique RNA-binding protein has any association with the process of liver stem cell differentiation in adults is of considerable interest. In this study, we investigated the expression of Musashi1 in adult liver stem-like cells that regulates the Notch signaling. Our results suggest a possible association of Musashi1 in liver stem-like cell differentiation.

METHODS

Liver stem cell line and culture

AN ESTABLISHED HEPATIC epithelial stem-like cell (HSLC) line derived from the healthy liver of adult male Sprague–Dawley rats[8] was used for experiments *in vitro*. This cell line has an immature liver cell phenotype with positive expression only for α-fetoprotein and negative for both albumin and cytokeratin (CK)19, and exhibits the potential to differentiate into cells of the hepatocytic lineage and serve as stem-like cells for differentiated hepatocytes.[8] The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C. Spheroidal aggregates of hepatocytes are known to exhibit higher functions than hepatocytes produced by monolayer culture.[9,10] In order to demonstrate the differentiation of HSLC into cells of the hepatocytic lineage *in vitro*, the cells were co-cultured for 24 h with epidermal growth factor (EGF) at a concentration of 10 ng/mL. These cells formed spheroids in culture. The expression of albumin was examined as a marker of cell differentiation in culture cells. The mRNA expression profile for both CK19 and tyrosine aminotransferase (TAT) was also examined in HSLC before and after culturing with EGF. Primary cultured, normal adult hepatocytes were used as control cells.[11]

Western blot analysis of albumin expression in HSLC

Expression of albumin was analyzed in HSLC cultured with or without EGF. The proteins were prepared by treating the cells with cell lysis buffer, followed by centrifugation. A 15-μg sample of proteins was subjected to a 10% sodium dodecylsulfate polyacrylamide ready gel (Bio-Rad Laboratories, Richmond, CA, USA). Resolved proteins were transferred electrophoretically to an Immunobilon-P membrane (Millipore, Bedford, MA, USA) at 4°C and processed for immunodetection. After blocking with 5% nonfat milk for 1 h at room temperature, the membrane was incubated with rabbit antirat albumin antibody (1:200 dilution; Cappel, Aurora, OH, USA) at 37°C for 2.5 h. The membrane was then incubated with alkaline phosphatase-labeled goat antirabbit immunoglobulin (Ig)G antibody (1:1000 dilution; KPL, Gaithersburg, MD, USA) for 1.5 h at room temperature. Detection of the immunoreaction was performed with the BCIP/NBT phosphate substrate system (KPL), according to the manufacturer's protocol.

Reverse transcription polymerase chain reaction (RT–PCR)

The mRNA expression of *CK19*, *TAT*, *Notch* family (*Notch1* and *Notch2*) and its ligand *Jagged1*, and *Hes1* in both HSLC cultured with or without EGF and in primary cultured mature hepatocytes were examined by RT–PCR according to the procedure we previously described.[7] The PCR consisted of 35 cycles at a denaturation temperature of 94°C for 30 s, an annealing temperature of 58°C for 2 min and an extension temperature of 72°C for 1 min using a Perkin-Elmer 9600 thermal cycler platform (Perkin-Elmer, Norwalk, CT, USA). The primers for PCR to detect mRNA expression were: *Musashi1*, 5′-GGC TTCGTCACTTTCATGGACCAGGCG-3′ and 5′-GGGACC TGGTAGGTGTAAC-3′ (PCR product; 542 bp); *Hes1*, 5′-CCACTGCTACCCGTAAAGTC-3′ and 5′-GGCCTGAG GCTCTCAGTTCC-3′ (228 bp); *Notch1*, 5′-GACTATGCC TGCAGCTGTGCC-3′ and 5′-GGCTGCAGGGCACGTA GG-3′ (421 bp); *Notch2*, 5′-ATGTGTGTTACCTACCA CA-3′ and 5′-CCACAGTGGTACAGGTACTT-3′ (371 bp); *Jagged1*, 5′-CATCATAGCCTGTGAGCCTTC-3′ and 5′-ATATCATCCTCTTCCACTTCC-3′ (492 bp); CK19, 5′-TT GCGCGACAAGATTCTTGG-3′ and 5′-CATCTCACTCAG GATCTTGG-3′ (361 bp); and TAT, 5′-TGAACAGCAC TACCACTGTG-3′ and 5′-AGGCATCCTCCGTCTTCT GC-3′ (380 bp). The PCR reaction for β-actin was performed as an internal control (191 bp).[7]

Quantitation of Musashi1 mRNA levels in HSLC before and after differentiation

The total cellular RNA extracted from Hep3B cells positive for *Musashi1* mRNA expression[7] was used as a standard. The methods for RNA isolation and cDNA amplification were performed as previously.[7] To quantitate *Musashi1* mRNA levels in HSLC before and after differentiation, real-time PCR was performed using a LightCycler quick system 350S (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. The primers for detection of *Musashi1* mRNA in the real-time PCR were 5′-GGCTTCGTCACTTTCATGGA CCAGGCG-3′ and 5′-GGGACCTGGTAGGTGTAAC-3′. Quantitation test was performed in quadruplicate and the results were expressed as mean ± standard error (SE). Differences at $P < 0.05$ by Mann–Whitney *U*-test were considered significant.

Immunofluorescence staining for Musashi1 in HSLC

Expression of Musashi1 was analyzed by indirect immunofluorescence staining in HSLC cultured with or

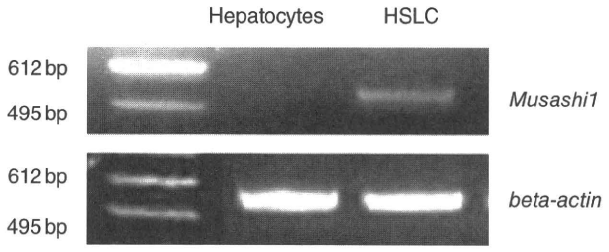

Figure 1 Reverse transcription polymerase chain reaction (RT–PCR) analysis of *Musashi1* mRNA expression in hepatic stem-like cells (HSLC) and primary cultured mature hepatocytes. The mRNA expression of *Musashi1* was detected in HSLC but not in hepatocytes. The predicted size of the PCR-amplified *Musashi1* product was 542 bp.

without EGF. A polyclonal rabbit anti-Musashi peptide antibody (Chemicon International, Temecula, CA, USA) that recognizes human and rodent Musashi1 was used as a primary antibody. Fluorescein isothiocyanate-labeled $F(ab')_2$ fragments of goat antirabbit IgG (Dako-Cytomation, Kyoto, Japan) were used as a secondary antibody. The cells were examined with the aid of a fluorescence microscope.

RESULTS

Expression of Musashi1 mRNA and Musashi1 protein in HSLC

THE MRNA EXPRESSION of *Musashi1* was detected in HSLC but not in primary cultured mature hepatocytes by RT–PCR (Fig. 1). The RT–PCR product of the *Musashi1* mRNA amplified using specific primers predicted a band of 542 bp. Protein expression of Musashi1 was detected in HSLC by immunofluorescence staining (Fig. 2), but it was not detected in primary cultured hepatocytes.

Downregulated expression of Musashi1 mRNA and Musashi1 protein in differentiated HSLC producing albumin

Hepatic stem-like cells were cultured with 10 ng/mL EGF for 24 h and harvested when they formed spheroids. Albumin expression in these cells was examined as a marker of cell differentiation from an immature to a mature state. Albumin expression was not detected in HSLC before culturing with EGF, but was detectable by western blot analysis after culturing with EGF. Quantitative analysis revealed that the level of *Musashi1* mRNA in the differentiated HSLC was significantly lower than that of original HSLC ($1.06 \pm 1.34 \times 10^{10}$ copies/mL vs $4.33 \pm 2.68 \times 10^{13}$ copies/mL, mean ± SE, $P < 0.05$) (Fig. 3). Expression of Musashi1 protein was not detected in differentiated HSLC producing albumin by immunofluorescence staining.

Changes in mRNA expression of the Notch signaling markers in HSLC differentiation

Because the expression of *Musashi1* mRNA was found to be downregulated in the cell differentiation process, the expression of the *Notch* family mRNA was investigated in HSLC before and after culturing with EGF by RT–PCR analysis. *Notch1* mRNA expression was detected in HSLC before and after culturing with EGF, but its expression was not detected in primary cultured mature hepatocytes. *Notch2* mRNA expression was found in HSLC before and after culturing with EGF as well as in primary cultured mature hepatocytes. The notch ligand *Jagged1* mRNA expression was detected in HSLC before and after culturing with EGF, but its expression was not detected in primary cultured mature hepatocytes. *Hes1* mRNA expression was detected in the original HSLC, but not in those producing albumin after culturing with EGF, and nor was it detected in primary cultured mature hepatocytes. The biliary cell marker, CK19 mRNA expression was not detected in any cells examined. The hepatocyte marker, TAT mRNA expression was not detected in the original HSLC, but its expression was detectable in those

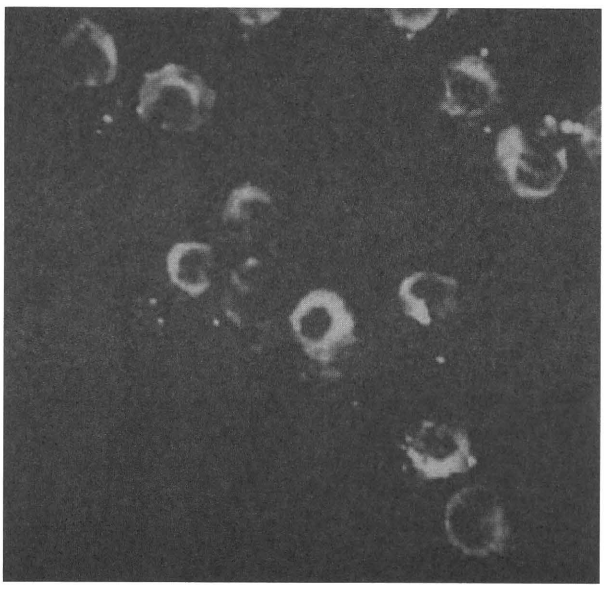

Figure 2 Immunofluorescence staining for Musashi1 protein in the cytoplasm of hepatic stem-like cells (original magnification ×400).

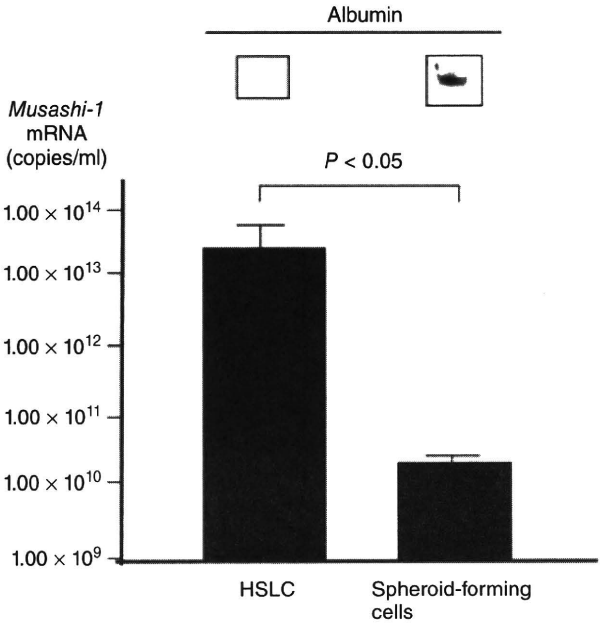

Figure 3 Western blot analysis of albumin expression in hepatic stem-like cells (HSLC). Albumin expression was not detected in the original HSLC, but was detected in spheroid-forming cells after culturing with epidermal growth factor (EGF). Real-time polymerase chain reaction analysis of *Musashi1* mRNA expression in HSLC before and after culturing with EGF. The level of *Musashi1* mRNA in the spheroid-forming differentiated cells was significantly lower than that of original HSLC. Quantitation test was performed in quadruplicate.

producing albumin after culturing with EGF as well as in primary cultured mature hepatocytes (Fig. 4).

DISCUSSION

ALTHOUGH A CLOSE association has been shown to exist between Musashi1 and Notch signaling in neural stem cell differentiation,[4] the involvement of such a mechanism in the differentiation of stem cells in digestive organs has not been fully elucidated. Recently, it was shown that Musashi1 is expressed in putative intestinal stem cells [12] and can be used as a marker of stem cells and early-lineage progenitor cells in murine intestinal tissue.

In this study, we have demonstrated that Musashi1 is expressed in putative rat liver stem-like cells at the mRNA and protein level. Interestingly, the mRNA expression of *Hes1* was downregulated along with *Musashi1* mRNA expression in the differentiated cells that produced albumin. Notch proteins were initially identified in *Drosophila* and *Caenorhabditis elegans*, but have subsequently been identified in vertebrate species.[13] It has been reported that there is an association between expression of the *Notch* family, and bile duct formation,[14] liver cell regeneration after partial hepatectomy[15] and neovascularization in the human diseased liver,[16] although no such association has been demon-

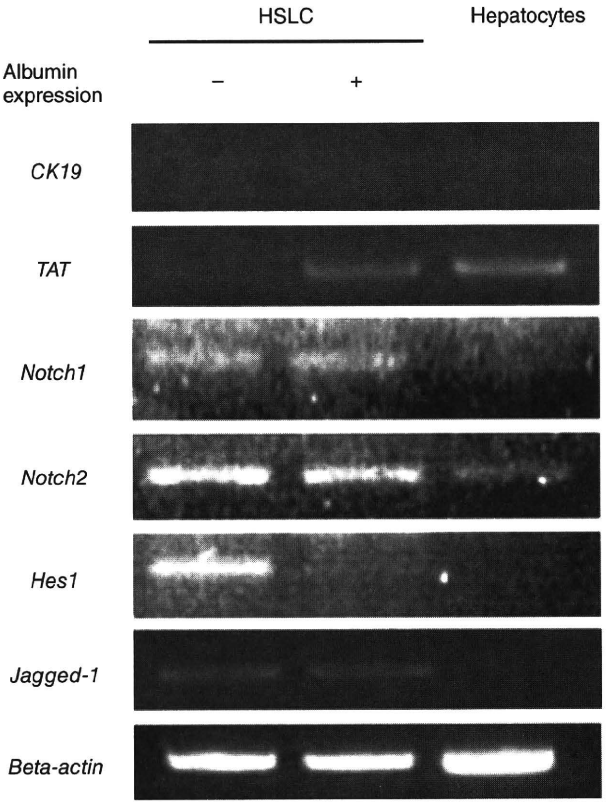

Figure 4 The mRNA expression for cytokeratin (CK)19, tyrosine aminotransferase (TAT) and *Notch* family in hepatic stem-like cells (HSLC), as revealed by reverse transcription polymerase chain reaction (RT-PCR) analysis. CK19 mRNA expression was not detected in all cells. TAT mRNA expression was not detected in the original HSLC, but it was detectable in differentiated cells producing albumin as well as in mature hepatocytes. The mRNA expression for *Notch1* and *Jagged1* was detected in HSLC, but it was not detected in mature hepatocytes. *Notch2* mRNA expression was detected in all cells. The mRNA expression of *Hes1* was detected in HSLC, but was undetectable in differentiated cells producing albumin as well as in mature hepatocytes. The predicted size of the PCR-amplified product was 361 bp for CK19, 380 bp for TAT, 421 bp for *Notch1*, 371 bp for *Notch2*, 492 bp for *Jagged-1* and 228 bp for *Hes1*.

strated in liver stem cells. Both Notch-1 and its ligand Jagged-1 have been detected in the hepatic progenitor cells, referred to as oval cells, in the liver of the 2-acetylaminofluorene 70% hepatectomy models.[17] In this study, *Notch1* and *Jagged1* mRNA expressions were detectable in HSLC , but not in mature rat hepatocytes. The absence of Notch1 expression in mature hepatocytes has also been demonstrated in humans.[17] *Hes1* mRNA expression is activated by a nuclear translocation of the Notch intracellular domain.[11] In the present study, we could show that *Hes1* mRNA expression was also detectable in HSLC at a location downstream of this signaling. The mRNA expression of *Notch1–Hes1* signaling was upregulated in Musashi1-positive HSLC and was undetectable in the differentiated cells producing albumin. To confirm the association of Musashi1 with an activation of the Notch signaling, it would be important to see if changes in Musashi1 expression level by the gene knockdown influence of liver stem-like cell differentiation. In addition, expression of Musashi1 in the liver tissue remains unknown. Further studies are needed to elucidate these issues.

The roles of Musashi1 in the development of liver morphology and function remain unknown. A report on the *Musashi1* gene disruption model revealed that homozygous newborn mice are not prone to immediate death, but frequently develop obstructive hydrocephalus with aberrant proliferation of ependymal cells.[5] As Musashi2, another member of the RNA-binding protein family,[18] is co-expressed in this model, gene compensation of *Musashi2* in the *Musashi1* disruption model might contribute to organ development, and hence improve the chances of survival. Analyses of alteration of liver-specific mRNA expression as well as liver morphology in such a model would provide information that could extend our understanding of the role of Musashi1 in the development of liver morphology and function.

In conclusion, the results of this study suggest that Musashi1 is expressed with Notch signaling markers in liver stem-like cells as well as in neural stem cells in adults. The role of Musashi1 in liver regeneration warrants further investigation.

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