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ScienceDirect
Neuroscience Letters 429 (2007) 43–48
Neuroscience Letters
www.elsevier.com/locate/neulet

Neural-specific ablation of the scaffold protein JSAP1 in mice causes neonatal death

Asuka Iwanaga [a,1], Tokiharu Sato [a,1], Kazushi Sugihara [b], Atsushi Hirao [c], Nobuyuki Takakura [d], Hiroshi Okamoto [e], Masahide Asano [b], Katsuji Yoshioka [a,*]

[a] *Division of Molecular Cell Signaling, Department of Molecular and Cellular Biology, Cancer Research Institute, Kanazawa University, Ishikawa 920-0934, Japan*
[b] *Division of Transgenic Animal Science, Advanced Science Research Center, Japan*
[c] *Division of Molecular Genetics, Center for Cancer and Stem Cell Research, Cancer Research Institute, Kanazawa University, Japan*
[d] *Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*
[e] *Department of Advanced Biological Sciences for Regeneration (Kotobiken Medical Laboratories), Tohoku University Graduate School of Medicine, Sendai, Japan*

Received 29 August 2007; received in revised form 25 September 2007; accepted 26 September 2007

Abstract

We previously identified c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase-associated protein 1 (JSAP1, also known as JNK-interacting protein 3) as a scaffolding factor for JNK intracellular signaling pathways. Targeted gene-disruption studies have shown that JSAP1-null mice are unable to breathe and die shortly after birth. Although neural defects might be responsible for their death, there has been no convincing evidence for this. Here we first generated genetically engineered mice carrying a loxP-flanked (floxed) *jsap1* gene. To evaluate the validity of this deletion as a *jsap1* conditional knockout (KO), we created mice in which the same exon was deleted in all cell lineages, and compared their phenotypes with those of the *jsap1* conventional KO mice reported previously. The two KO lines showed indistinguishable phenotypes, i.e., neonatal death and morphological defects in the telencephalon, indicating that the conditional deletion was a true null mutation. We then introduced the floxed *jsap1* deletion mutant specifically into the neural lineage, and found that the *jsap1* conditional KO mice showed essentially the same phenotypes as the JSAP1-null mice. These results strongly suggest that the neonatal death of *jsap1*-deficient mice is caused by defects in the nervous system.

Keywords: Conditional knockout mouse; MAP kinase; Signal transduction

Mitogen-activated protein kinase (MAPK) intracellular signal transduction pathways are protein kinase cascades, and are well conserved from yeast to humans [9,24]. MAPK signaling modules consist of three distinct kinases, namely a MAPK kinase kinase (MAP3K), a MAPK kinase (MAPKK), and a MAPK. MAPK is phosphorylated and activated in response to a variety of extra- and intracellular stimuli; this phosphorylation is mediated by MAPKK, which in turn is phosphorylated and activated by MAP3K. In mammals, three groups of MAPK cascades, i.e., the extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase [JNK, also known as stress-activated protein kinase (SAPK)] cascades, have been extensively studied. These mammalian MAPK cascades play pivotal roles in multiple cellular processes, including proliferation, differentiation, apoptosis, and migration [6,16,25]. Furthermore, a number of MAPK signaling components form signaling networks in cells. Scaffold proteins of the mammalian MAPK cascades are implicated in the spatio-temporal regulation of these pathways by organizing the MAPK signaling components into functional modules (reviewed in [7,18,27]). The scaffolding complexes enable the efficient activation of specific MAPK cascades as well as the protection of the components within the relevant MAPK modules from undesired activation by other signaling molecules in cells.

We previously identified JNK/SAPK-associated protein 1 [JSAP1, also known as JNK-interacting protein 3 (JIP3)] as a scaffold protein for mammalian JNK MAPK cascades [11,14].

* Corresponding author. Tel.: +81 76 234 4532; fax: +81 76 234 4532.
E-mail address: katsuji@kenroku.kanazawa-u.ac.jp (K. Yoshioka).
[1] These authors contributed equally to this study.

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doi:10.1016/j.neulet.2007.09.057

In mice, JSAP1 is expressed highly in the brain and at low or moderate levels in other tissues, such as heart, lung, and testis [1,3,10,11,14,17]. *In vitro* differentiation studies with JSAP1-null mouse embryonic stem (ES) cells showed that JSAP1 is important in embryonic neurogenesis and cardiomyogenesis [21,26]. Furthermore, targeted disruption of the mouse *jsap1* gene resulted in morphological defects in the brain, such as the lack of the telencephalic commissure, and neonatal death [8,13]. Although neural abnormalities might be responsible for the death of the JSAP1-null mice, there has been no convincing evidence for this. In the present study, we generated and analyzed neural-specific *jsap1* KO mice.

All the experiments involving animals were conducted according to guidelines for the care and use of laboratory animals of Kanazawa University, with the approval of the Committee on Animal Experimentation of Kanazawa University.

The targeting vector used to generate the triple-loxP allele of the *jsap1* gene was described previously [21]; in that study, the vector was used to generate *jsap1*-deficient Nkx2.5/green fluorescent protein (GFP) mouse embryonic stem (ES) cells. The targeting vector was linearized with NotI, and gene targeting using 129/Ola E14-1 ES cells was performed as described previously [2]. We identified four targeted clones by polymerase chain reaction (PCR) and Southern blotting with the 5′-flanking probe shown in Fig. 1A, of which two were used for further experiments. The two independent ES clones contained all three loxP sites in the locus and gave rise to germ-line chimeras

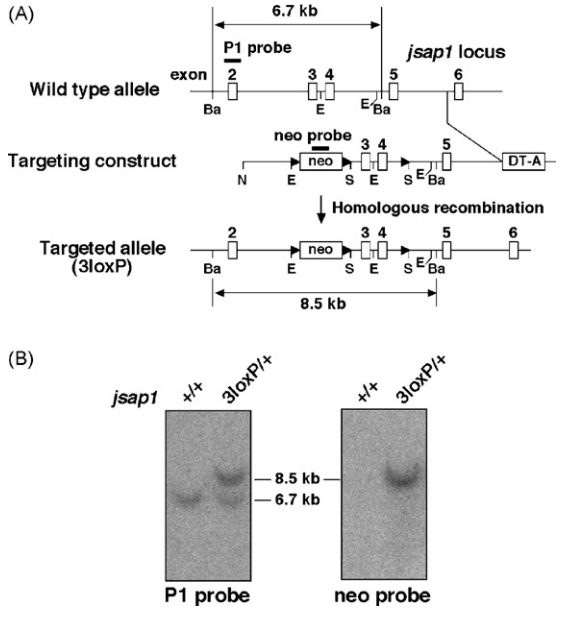

Fig. 1. Generation of a floxed allele of the mouse *jsap1* gene. (A) Schematic illustration of the targeting of the *jsap1* gene. The wild-type allele (*jsap1*$^{+}$) and targeted allele (*jsap1*^{3loxP}) are shown together with the targeting construct. Ba, BamHI; E, EcoRI; N, NotI; S, SalI; neo, neomycin-resistance cassette; DT-A, *diphtheria* toxin-A gene; arrowhead, loxP site. (B) Southern blot analysis of *jsap1*$^{+/+}$ (+/+) and *jsap1*$^{3loxP/+}$ (3loxP/+) mice. Genomic DNA isolated from the mice was digested with BamHI, and subjected to Southern blotting with the 5′-flanking P1 fragment (left panel) and the neo fragment (right panel) indicated in (A) as probes. The wild-type and targeted *jsap1* loci gave rise to 6.7-kb and 8.5-kb bands, respectively, using the P1 probe. The neo probe detected only the 8.5-kb band derived from the targeted allele.

by the aggregation method [2]. The resultant male chimeras were mated with female Cre-transgenic mice, which transiently express Cre recombinase in their eggs [19]. Some of the newborn mice were found to carry the deleted allele lacking both *jsap1* exons 3 and 4 as well as the neomycin-resistance (neo) cassette (*jsap1*$^{-}$); others carried loxP-flanked (floxed) *jsap1* exons 3 and 4, but not the neo cassette (*jsap1*^{flox}). These mutant mice were backcrossed to C57BL/6J for three generations, and the resulting mice were used in this study unless indicated otherwise. For PCR genotyping, three primers were used: 5′-GCCCCATGAGACCTCTGATACTTGCTC-3′ (primer 1), 5′-GCACAGCAGCCTGACAGAAAGAAGTAC-3′ (primer 2), and 5′-AGGAACAGCCATAGATGGGACCGT-3′ (primer 3) (nucleotides 60386–60412, 60953–60979, and 62518–62541 in GenBank accession no. AF220294, respectively). Homozygous (*jsap1*$^{flox/flox}$ and *jsap1*$^{-/-}$) mice were obtained by intercrossing the respective heterozygous (*jsap1*$^{flox/+}$ and *jsap1*$^{+/-}$) mice. A transgenic mouse strain expressing Cre recombinase under the control of the *nestin* promoter [23] was obtained from the Jackson Laboratory. The characteristic expression pattern of the transgene in the nestin-Cre mouse strain was confirmed using a GFP reporter mouse strain [12]. Noon of the day on which the vaginal plug was found was counted as embryonic day (E) 0.5. The postnatal day (P) was counted as described.

The brains of E18.5 embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) overnight at 4 °C and processed in paraffin wax following standard procedures. The paraffin-embedded sections (7-μm thick) were dewaxed and subjected to hematoxylin and eosin (HE) or Nissl staining.

Whole brains and hearts of E18.5 embryos were lysed with RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate) containing Protease Inhibitor Cocktail (Sigma–Aldrich). The protein samples were subjected to immunoblotting using antibodies to JSAP1 (0.2 μg/ml [17]) and α-tubulin (1:5000, T5168; Sigma–Aldrich), as described previously [22]. Protein bands were visualized by the ECL Plus chemiluminescence system (GE Healthcare).

The brain, heart, lungs, and liver were removed from E18.5 embryos, fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4 °C, submerged in 30% sucrose, embedded, and sectioned using a cryostat. The sections (20-μm thick) were blocked with 2% bovine serum albumin, 2% goat serum, and 0.4% TritonX-100 at room temperature for 1 h. The sections were then incubated overnight at 4 °C with the anti-JSAP1 antibody (1 μg/ml) followed by a 1-h incubation with Alexa488-labeled secondary antibody (1:1000, A11008; Invitrogen). TOTO-3 iodide (Invitrogen) was used as a nuclear counterstain. Photographs were taken with a confocal laser scanning microscope (Zeiss, LMS510).

We constructed a targeting vector containing three loxP sites in the same orientation, in which a neo cassette flanked by two loxP motifs was placed upstream of exon 3 and a third loxP site was placed downstream of exon 4 of the *jsap1* gene (Fig. 1A). Cre-mediated recombination of the targeted allele results in a frameshift mutation if the flanking exons fortuitously splice,