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Impairment of cardiomyogenesis in embryonic stem cells lacking scaffold protein JSAP1

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Abstract

We previously reported that c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase-associated protein 1 (JSAP1), a scaffold protein for JNK signaling, is important in embryonic stem (ES) cells during neurogenesis. In that study, we also observed the altered expression of mesodermal marker genes, which indicated that JSAP1 is involved in the differentiation of mesodermal lineages. Here, we investigated the function of JSAP1 in cardiomyocyte development using JSAP1-null ES cells, and found that cardiomyogenesis was impaired in the JSAP1-null mutant. The JSAP1 deficiency resulted in lower gene expression of the cardiac transcription factor Nkx2.5 and contractile proteins. In contrast, the mutant showed a significantly higher expression of mesoderm-related markers other than those of the cardiomyocyte lineage. Together, these results suggest that JSAP1 may be important for the differentiation of the mesodermal lineages, functioning as a positive factor for cardiomyocyte differentiation, and as an inhibitory factor for differentiation into other lineages.

Keywords: Cardiomyocyte; Embryoid body; ES cells; Gene disruption; JNK; JSAP1; Mesoderm; Nkx2.5; Scaffold protein

Embryonic stem (ES) cells possess pluripotency and differentiate spontaneously into various specialized cell types, including cardiomyocytes, when cultivated in vitro as embryoid bodies (EBs) [1]. Thus, ES cells provide a useful model system for studying the molecular mechanisms that regulate cardiomyocyte differentiation. In addition, because ES cells could represent an unlimited source of functionally intact cardiomyocytes, elucidation of the mechanisms of ES cell-derived cardiomyogenesis is important from the viewpoint of regenerative medicine [1,2]. Extensive studies in vivo and in vitro have led to the identification of signaling molecules, such as bone morphogenesis proteins (BMPs), fibroblast growth factors (FGFs), and Wnts, as key molecules in cardiomyogenesis [1,3–6]. Although these secreted proteins interact with their cognate receptors to modulate the expression of cardiac genes, such as *Nkx2.5* and *Gata4*, it remains to be elucidated how the intracellular signaling pathways regulate cardiomyogenesis at the molecular level.

Mammalian MAP kinase (MAPK) signal transduction pathways, consisting of a MAPK, MAPKK, and MAPKKK, play important roles in many cellular processes, including proliferation, differentiation, and apoptosis

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[7,8]. Scaffold proteins of the mammalian MAPK cascades are thought to function in the spatial and temporal regulation of these pathways by organizing MAPK signaling components into functional modules [9,10]. These functional complexes enable the efficient signal transduction of specific MAPK cascades. 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 (JIP3), as a scaffold protein for JNK MAPK cascades [11,12]. Furthermore, our group and others reported that JSAP1 is required for embryonic neurogenesis [13–15]. During the course of the analyses with *Jsap1*-deficient mouse ES cells, we observed the altered expression of several genes, including the mesodermal marker *Bmp4*, indicating that JSAP1 is also involved in mesoderm-related cell lineages [14].

In the present study, we examined the function of JSAP1, focusing on the cardiomyocyte differentiation of ES cells. For this purpose, we used a previously established mouse ES cell line, Nkx2.5GFP ES, in which the green fluorescent protein (GFP) gene is knocked into one of the *Nkx2.5* loci [16]. This cell line allows us to track Nkx2.5-positive cell lineages by using GFP as a reporter. We generated *Jsap1*-deficient Nkx2.5GFP ES cells and investigated their cardiomyogenesis. Our data suggest that JSAP1 plays an important role in the differentiation process of ES cells into the cardiomyocyte lineage.

Materials and methods

Mouse ES cell culture and differentiation. The culture and differentiation induction of Nkx2.5GFP ES cells were performed as described previously [16].

Generation of Jsap1-deficient Nkx2.5GFP ES clones. Mouse *Jsap1* genomic clones were isolated from a lambda 129/Sv genomic library (Stratagene) using mouse JSAP1 cDNA as the probe. The 9.1-kb genomic region, from 0.23-kb downstream of exon 2 to 0.39-kb upstream of exon 6 of the *Jsap1* gene, was used to construct the targeting vector (Fig. 1A). The left and right arms of the targeting vector were 2.7 and 6.4 kb, respectively. A neomycin-resistance cassette [17,18], the PGK promoter-driven neomycin-resistance gene with loxP sites at both ends, was inserted between the left and right arms. A third loxP site from plasmid pULwL [19] was inserted 0.64-kb downstream of exon 4 in the right arm. The *diphtheria* toxin-A (DT-A) gene [20] was introduced for negative selection. The targeting vector was linearized with *Not*I, and gene targeting using Nkx2.5GFP ES cells was performed as described previously [21]. Homologous recombinants were identified by polymerase chain reaction (PCR) and by Southern blotting. Subsequently, Cre recombinase was introduced transiently as the pCre-pac plasmid [22] by electroporation of the appropriately targeted ES cell clones, which were then selected for 72 h in 1 μg/ml puromycin to obtain clones with a fully deleted *Jsap1* allele. A second round of homologous recombination and Cre-mediated recombination was carried out as described above, to generate $Jsap1^{-/-}$ Nkx2.5GFP ES cell clones.

Western blot analyses. Cultured cells were lysed with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% glycerol, and 1% SDS) containing Protease Inhibitor Cocktail (Sigma). The protein samples were subjected to Western blot analysis as described previously [23]. The anti-JSAP1 polyclonal antibody (Ab) was described previously [24]. The anti-JNK polyclonal Ab and anti-phospho-specific JNK monoclonal Ab were purchased from Cell Signaling, and the anti-α-tubulin monoclonal Ab was obtained from Sigma.

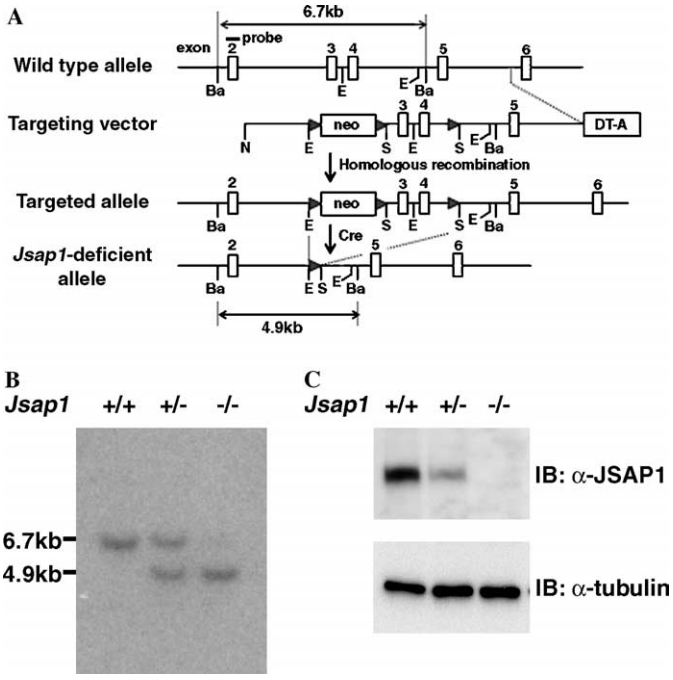

Fig. 1. Generation of JSAP1-null Nkx2.5GFP ES cells. (A) Strategy for the targeted disruption of the *Jsap1* gene in Nkx2.5GFP ES cells. The wild-type allele ($Jsap1^{+}$), targeted allele ($Jsap1^{3loxP}$), and *Jsap1*-deficient allele ($Jsap1^{-}$) are shown together with the targeting vector. $Jsap1^{-}$ was generated from $Jsap1^{3loxP}$ using Cre recombinase. A second round of homologous recombination and the subsequent Cre-mediated recombination gave rise to $Jsap1^{-/-}$. Ba, *Bam*HI; E, *Eco*RI; N, *Not*I; S, *Sal*I; neo, a neomycin-resistance cassette; DT-A, the *diphtheria* toxin-A gene; arrowhead, loxP site. (B) Southern blot analysis of $Jsap1^{+/+}$ (+/+), $Jsap1^{+/-}$ (+/−), and $Jsap1^{-/-}$ (−/−) ES cells. Genomic DNAs (1.5 μg/lane) isolated from the ES cells were digested with *Bam*HI and subjected to Southern blotting with the 5′-flanking 0.6-kb fragment indicated in (A) as a probe. (C) Cell lysates (30 μg/lane) from the ES cells were analyzed by immunoblotting with an anti-JSAP1 antibody (upper panel). The positions of protein size markers are indicated on the left. The expression of α-tubulin is shown as a loading control (lower panel).

Real-time reverse transcription (RT)-PCR. Total RNAs were prepared from EBs by using Sepasol RNAI (Nakalai Tesque) according to the manufacturer's instructions, and the RNA samples were treated with RNase-free DNaseI (Promega) to eliminate genomic DNA contamination. Real-time RT-PCR was performed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) with the SYBR RT-PCR Kit (Takara). The expression level of each mRNA was normalized with respect to that of *Gapdh* mRNA. The following primers were used for the RT-PCR analysis of *Bmp4* and *Brachyury* mRNAs: 5′-GTTTC CTCTTCAACCTCAGC-3′ and 5′-TATACGGTGGAAGCCCTGTT-3′ (for *Bmp4*), and 5′-CTCACCAACAAGCTCAATGG-3′ and 5′-ACAGC TATGAACTGGGTCTC-3′ (for *Brachyury*). The other gene-specific primers were described previously [16].

Fluorescence-activated cell sorting analysis. Fluorescence-activated cell sorting (FACS) analysis was carried out as follows, using phycoerythrin (PE)-conjugated monoclonal Abs to Flk1 (Pharmingen), CD31 (eBioscience), and CD45 (eBioscience): floating EBs (64 EBs per each FACS experiment) were dissociated with 0.25% trypsin and 1 mM EDTA (Invitrogen) at 37 °C for 1–3 min with occasional agitation. As soon as the solution became cloudy, serum-containing medium was added to neutralize the trypsin. After centrifugation, the single cells were resuspended in PBS containing 2% serum and 1 μg/ml 7-amino actinomycin D (7AAD), and analyzed with a FACS Calibur (BD Bioscience).