In Vitro Development of Mouse Embryonic Stem Cells Lacking JNK/Stress-activated Protein Kinase-associated Protein 1 (JSAP1) Scaffold Protein Revealed Its Requirement during Early Embryonic Neurogenesis*

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The Jsap1 gene encodes a scaffold protein for c-Jun N-terminal kinase cascades. We established c-Jun N-terminal kinase (JNK)/stress-activated protein kinase-associated protein 1 (JSAP1)-null mouse embryonic stem cell lines by homologous recombination. The JSAP1-null embryonic stem cells were viable, however, exhibited hyperplasia of the ectoderm during embryoid body formation, and spontaneously differentiated into neurons more efficiently than did wild-type cells. The expression of components of c-Jun N-terminal kinase cascades and a subset of marker mRNAs during early embryogenesis was altered in the JSAP1-null mutants. Retinoic acid dramatically increased the expression of JSAP1 and JNK3, which were co-precipitated with anti-JNK3 in the neuroectoderm of wild type but not JSAP1-null embryoid bodies. In the neurons differentiated from the wild type embryoid bodies, JSAP1 was localized in the soma, neurites, and growth cone-like structure of the neurites, and neurite outgrowth from the JSAP1-null embryoid bodies was apparently less efficient than from wild type. JSAP1 and c-Jun N-terminal kinase 3 were coexpressed in the embryonic ectoderm of E7.5 mouse embryos, whereas Wnt1 and Pax2 were coexpressed with JSAP1 at the midbrain-hindbrain junction in E12.5 mouse embryos, thus suggesting that JSAP1 is required for early embryonic neurogenesis.

The mitogen-activated protein kinase (MAPK)3-signaling pathway is an intracellular cascade consisting of MAPK, MAPK kinase, and MAPK kinase kinase (1, 2). In mammals, at least four groups of MAPKs, extracellular signal-regulated kinases 1/2, p38 proteins, extracellular signal-regulated kinase 5, and the c-Jun N-terminal kinases (JNKs) (also known as stress-activated protein kinases), have been intensively characterized (3, 4).

The JNK pathway of MAPKs is primarily activated in response to extracellular stimuli such as cytokines, heat shock, or radiation and mediates the regulations of cell proliferation, apoptosis, tumorogenesis, and embryonic morphogenesis. Three genes encoding JNK were identified in mammals; the Jnk1 and Jnk2 genes are ubiquitously expressed, and the Jnk3 gene is specifically expressed in the brain, heart, and testis in adults. Mice lacking only one of three genes or Jnk1 and Jnk3 or Jnk2 and Jnk3 develop normally and are viable; however, the double-knockout mice of Jnk1 and Jnk2 are lethal during early embryogenesis at mid-gestation with a defect of neural tube closure (5), thus demonstrating a functional redundancy of the JNKs during embryogenesis. In both Drosophila and Xenopus, the JNK signal pathway has also been reported to mediate embryonic morphogenesis through regulating the epithelial movement and planar cell polarity during gastrulation (6, 7).

It has been shown that the MEKK1 and MLKs, as MAPK kinase kinases, selectively activate the JNK cascade (8) through the phosphorylation of MAPK kinases such as MAPK kinase-4 (MKK4, also known as SEK1) and MKK7, which specifically phosphorylate JNKs. Once activated, the JNKs phosphorylate several transcription factors such as c-Jun, Jun-D, ATF2, and Elk1, which in turn regulate the transcription activity of many target genes. As well as JNK1/2-deficient mice, mice deficient in MKK4 or c-Jun are lethal at mid-gestation with a severe abnormality of liver formation (9, 10). MKK7 is also required for embryonic viability (11). A disruption of MEKK1 gene results in defective eyelid closure (12). It has, thus, been established that the JNK-signaling pathways play a critical role in embryogenesis.

In addition to MEKK1 and MLKs, other MAPK kinase kinases such as MEKK4, TAK1, and ASK1 can also activate the JNK and p38 cascades through activation of MKK4 and MKK7 for JNKs and MKK3 and MKK6 for p38s, whereas MEKK3 and TPL-2 can activate the extracellular signal-regulated kinase, p38, and the fourth extracellular signal-regulated kinase 5 cascades as well as the JNK cascade. These MAPK kinase kinases are, thus, highly promiscuous for the selection of...
MAPK kinase (3, 4). To maintain the signaling specificity, efficiency, and integrity among these MAPK cascades, protein kinase components of each signaling pathway have to be tightly organized both spatially and temporally.

Studies of yeast have established that the protein kinase components of the mating MAPK pathway interact with the scaffold protein Ste65 and that this interaction is essential for the formation of a functional signaling module, thus suggesting that such a scaffold protein may be essential for any MAPK pathway (13). Indeed, recent studies of the JNK signal transduction pathway also have led to the identification of potential scaffold proteins such as JNK-interacting protein 1 and 2, which are also known as islet brain 1 and 2, and JSAP1 (JNK-stress-activated protein kinase-associated protein 1, also known as JNK-interacting protein 3) (14–16). We and others have shown that JSAP1 may function as a scaffold protein for JNK cascades, in which the components were reconstituted by their overexpression in cultured cells (15–17).

JSAP1 is one of the known clustered genes in the t complex of the mouse chromosome 17 that exhibit random monoallelic expression (18). The t complex contains several critical loci affecting embryonic development, male fertility, and male transmission ratio distortion (19), such as Brachyury, an essential gene for the mesoderm differentiation of vertebrates (20, 21). It is, thus, likely that JSAP1 may play an important role during early embryogenesis. In the present study, we established JSAP1-null embryonic stem cell lines by homologous recombination to elucidate their in vitro differentiation capacity and obtained evidence suggesting that JSAP1 plays an important role in early embryogenesis, especially in neurogenesis.

**EXPERIMENTAL PROCEDURES**

**Anti-JSAP1 Antibody—Mouse Jasp1 cDNA fragment encoding its N-terminal region (115–504 amino acids) was subcloned into pET22b (+) (Novagen) to express Trx-His-S-JSA1p (115–504) (15). The Jasp1 cDNA fragment was also subcloned into pET8c:TrpE to express TrpE-JSA1p (115–504). Rabbit polyclonal antibodies against the fusion protein Trx-His-S-JSA1p (115–504) were prepared as previously described (22). The antibodies were purified with the aid of antigen-affinity columns (TrpE-JSA1p (115–504)-Sepharose and TrpE-Sepharose columns), and the purified antibodies were able to detect 0.1 ng of TrpE-JSA1p protein and, thus, were designated as anti-JSAP1.

**Generation of Jasp1-deficient Embryonic Stem Cell Clones—Genomic fragments of 2.8 and 5.45 kilobases flanking the exon 1 were used to generate a targeting construct in which a 0.5-kilobase region of the exon 1 and a part of intron 1 with the initiation codon and 0.4-kilobase region of the intron 1, including the initiation codon and 0.4-kilobase region of the intron 1, were replaced by a neomycin-resistant cassette, pol II-Neo-poly(A) cassette. To increase the frequency of the gene targeting, a pair of the herpes simplex virus-1 and -2 thymidine kinase cassette was placed flanking the Jasp1 genomic sequence in the targeting vector (23). CCE embryonic stem (ES) cells were electroporated with the Sall-linearized targeting vector as described (24). Colonies double resistant to G418 (250 μg/ml) (Sigma-Aldrich) and ganciclovir (5 μM) (Japan Syntechs, Tokyo, Japan) were selected and homologous recombinants (Jasp1<sup>+</sup>) were identified by a Southern blot analysis. The targeted allele of Jasp1 was designated as Jasp1<sup>tm1Yun</sup> or Jasp1<sup>−</sup>. Subsequently, homozygous Jasp1<sup>−</sup>-deficient clones (Jasp1<sup>−/−</sup>) were obtained by selection in the presence of higher concentration of G418 (1.5 and 2.0 mg/ml) (Sigma-Aldrich) and ganciclovir (5 μg/ml aprotinin, 1 μM leupeptin, 1 μM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 50 mM NaF, 12 mM β-glycerophosphate) and disrupted by sonication. Immunoprecipitation was performed as previously described (22) with a mouse anti-JSAP1 antibody against JNK3 (S5183, Sigma). JSAP1 in the precipitate was detected by Western blotting with anti-JSAP1.

**RT-PCR—** For the RT-PCR analyses, total RNA was prepared from ES cells or embryoid bodies using ISOGEN (Nippon Gene Co. Ltd., Toyama, Japan) and RT primers as previously described (22).

**Immunoprecipitation—** Cell lysates were homogenized in lysis buffer (20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 50 mM NaF, 12 mM β-glycerophosphate) and disrupted by sonication. Immunoprecipitation was performed as previously described (22) with an antibody against JNK3 (S5183, Sigma). JSAP1 in the precipitate was detected by Western blotting with anti-JSAP1. JNK3 was detected with a rabbit anti-JNK3 antibody (Santa Cruz). JSAP1 was detected with a rabbit anti-JSAP1 antibody (Novagen).

**Statistical Analysis—** The data are expressed as the mean ± S.D. All data were compared using an unpaired t test for data with gaussian distribution, the Welch test for data with non-gaussian distribution. Statistical significance between wild type and JSAP1-null mutant was accepted at a level of p < 0.001. The data were analyzed by multiple comparisons using the Holm-Sidak method. Comparison of the mRNA expression levels of all genes was performed using real-time PCR, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. Differences were considered statistically significant at a level of p < 0.05.
gancyclovir-resistant ES clones were identified as correctly targeted clones by Southern blot analyses (Fig. 1B).

To examine whether or not JSAP1 is essential for the viability of ES cells, Jasp11−/− ES cells (clones 16 and 50) were exposed to a high concentration of G418, and more than 70% of the colonies were isolated. About 30 and 50% of them were found to be homozygous (Jasp11−/−) (Fig. 1B). There was neither any detectable JSAP1 mRNA nor JSAP1 protein in the homozygous mutants, whereas the levels of JSAP1 mRNA and protein in the heterozygotes were ~50% of those of the levels observed in wild type ES cells (Fig. 1, C and D).

Two independent clones of Jasp1-null (Jasp1−/−) ES cells grew faster than did either wild type or parental Jasp1+/+ ES cells (Fig. 1E). The homozygous mutants were morphologically indistinguishable from the wild type and Jasp1+/− ES cells and expressed a normal level of cell surface-specific embryonic antigen-1, which is a phenotypic marker of undifferentiated ES cells (Fig. 1F) (29). Immunofluorescent microscopy with anti-JSAP1 revealed that all of wild type ES cells examined expressed a substantial level of JSAP1; however, about 15% of the Jasp1+/− ES cells expressed no detectable JSAP1 (Fig. 1G), thus supporting the random monoallelic expression of Jasp1 gene (18). Again, there was no detectable JSAP1 in the Jasp1-null ES cells.

**Poor Embryoid Body Formation and Altered Neurite Outgrowth from the Jasp1-null Mutant**—To explore whether or not Jasp1-null ES cells are still totipotent, we examined their differentiated phenotypes in vitro in comparison with those of wild type ES cells using an in vitro differentiation protocol (25, 30) (Fig. 2A). Jasp1-null (Jasp1−/−) ES cells formed smaller EBs than wild type in the absence of RA (4/4−), and the treatment of Jasp1-null EBs with RA (4/4+) resulted in the formation of much smaller EBs, determined by measuring the area of a cross-section for each EB (Fig. 2, B and C). The capacities of neural differentiation of each EB were also examined. From wild type EBs numerous neurites elongated especially when EBs were exposed to RA for 4 days (4/4+), whereas significantly poor neurite elongation or synapse formation was seen in the Jasp1-null EBs even with RA treatment. In contrast, Jasp1-null EBs tend to elongate longer neurites more efficiently than wild type in the absence of RA (4/4−) (Fig. 2, D and E).

**Altered Expression of Differentiation Markers during the In Vitro Differentiation of Jasp1-null Mutant**—Because the Jasp1-null EBs underwent abnormal in vitro differentiation, we next investigated the temporal expression pattern of various differentiation markers by semi-quantitative RT-PCR (Fig. 3A). The expression patterns of Gata4 and HNF1, which are essential for visceral endoderm differentiation (31, 32), were essentially similar during the differentiation of wild type and Jasp1-null ES cells with or without RA. However, the expression of transferrin mRNA, a target for Hnf3βy involved in the differentiation of definitive endoderm (33), was consistently higher in the late stage of Jasp1-null EBs than in wild type regardless of RA treatment.

The expression of Brachyury, a marker for the mesoderm (20), was significantly altered in Jasp1-null EBs. The expression of Brachyury was highly induced in the wild type EBs (4−) and was diminished by RA treatment (4+/4−). In contrast, in the early Jasp1-null EBs (4−), there was no detectable Brachyury mRNA, but the level tended to increased later with or without RA treatment (4−, 4+/4−). The expression of mRNAs for BMP4 and Nodal, which are members of transforming growth factor-β superfamily and involved in mesoderm induction and gastrulation (34), were not apparently altered throughout the in vitro differentiation both in the wild type and Jasp1-null mutants.

**Keratin 17** is a marker for epidermal cells (35), and its expression level was higher in the Jasp1-null mutants than wild type in all stages examined. The expression of both Emx2, which is essential for forebrain development (36), and Otx1, whose expression begins at the 1–3 somite stage in the anterior neuroectoderm of mouse embryo (37), was low in both undifferentiated wild type and Jasp1-null ES cells. Closely similar levels of Emx2 and Otx1 mRNA were detected in the early EBs (4−), which were formed from the two ES cell lines; however, the levels only highly increased in the late Jasp1-null EBs (4−) in the absence of RA. RA treatment, in contrast, increased the expression levels of Emx2 and Otx1 only in the wild type EBs. The expression of Emx2, Wnt1, Pax2, and Pax5, which are known to be involved in the regulation of mesencephalon and metencephalon development (38), was further examined. In the undifferentiated Jasp1-null but not wild type ES cells, only high level expression of Pax2 was detected among the four genes. In the early EBs (4−), the expression of Pax5 was increased in wild type EBs but not Jasp1-null EBs. During the formation of the late EBs in the absence of RA, the expression levels of Wnt1 and Pax5 were highly increased in the Jasp1-null EBs (4−), and RA treatment increased the expression levels of the four genes in the wild type but not in the Jasp1-null EBs. High levels of Wnt1 protein in the late EBs (4−), which were formed from wild type ES cells in the presence of RA, or in the late EBs, which were formed from the Jasp1-null mutants in the absence of RA, were confirmed by Western

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**TABLE I**

| Primer  | 5’ Sequence | 3’ Sequence |
|---------|-------------|-------------|
| Gata4   | TGTCAATTGTTGGGCGCATGTC | GTGCCCCACGCTTTTACCTTG |
| HNF1    | GAAACGAACGGAGATCCCAGGAGCATGTC | TAGGACTTACAGGCGCATTCAGG |
| transferrin | AGTTCTAAATGGTTGGAGGATTGTC | ACTGGTTCTCTGAGTTCATGTC |
| Brachyury | AGTTGAAGAGGGCTCATGAGC | GAACCAAGCTGAATGGAC |
| BMP4    | AGTTGCTCTCTGACACCATGTC | GCTGGAAGAGGGCTCATGAGC |
| Nodal   | TGAGAGCATGTCGAGTACGAGGAC | AGATTGCTCTCTGACACCATGTC |
| Keratin17 | TAGCTGGAAGAGGGCTCATGAGC | GAACCAAGCTGAATGGAC |
| En2     | TCTCGAGGACAGCATTTGATGGA | TAGCTGGAAGAGGGCTCATGAGC |
| Wnt1    | CTTTGGGAGATGCCTACCAAGGAC | GAACCAAGCTGAATGGAC |
| Pax2    | TAGGAGAAGGGAAGTATGGGAGGAC | GAACCAAGCTGAATGGAC |
| Pax5    | TAGGGAAGAGGGCTCATGAGC | GAACCAAGCTGAATGGAC |
| Jasp1   | CAGATGAGGACAGATGGGAC | GAACCAAGCTGAATGGAC |
| Jnk3    | CACGCTGGAAGAGGGCTCATGAGC | GAACCAAGCTGAATGGAC |
| c-Jun   | GAAGGAGATGAGGAGGAGGGAGGAC | GAACCAAGCTGAATGGAC |
| Aef2    | ACATGGGGAGGAGGAGGGAGGAC | GAACCAAGCTGAATGGAC |
| Ef1     | TGCGGAGGAGGAGGAGGGAGGAC | GAACCAAGCTGAATGGAC |
Fig. 1. Generation and characterization of JSAP1-null ES mutants. A, strategy for the targeted disruption of the Jsap1 gene. The upper lines represent the wild-type Jsap1 (Jsap1+/−) allele and a targeting vector, whereas the lower line shows the mutant Jsap1 (Jsap1−/−) allele. Restriction enzyme sites are indicated (A, Apal; B, BspI; Ba, BamHI; H, HindIII). kb, kilobase(s). B, Southern blot findings with the 3′-flanking P2 probe for BamHI-digested genomic DNA are shown. +/+ , CE (Jsap1+/+) ES cell line; +/− , Jsap1+/− ES cell lines (#16, #50); −/− , Jsap1−/− ES cell lines. Using the 5′-flanking P1 probes, the sizes of BspI-digested fragments were also confirmed in each ES cell clone (data not shown). C, RT-PCR analysis of the expression of the Jsap1 gene in targeted ES cells. Shown are Jsap1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels presented in the parental CCE ES cells (+/+), one of the heterozygous (+/−), and homozygous (−/−) Jsap1 mutant ES cells. D, Western blot analysis of JSAP1 expression in targeted ES cells. Shown are the JSAP1 and β-actin levels observed in the parental CCE ES cells (+/+), one of the heterozygous (+/−), and homozygous (−/−) Jsap1 mutant ES cells. E, the proliferation of the Jsap1 mutant ES cells. The parental CCE ES cells (+/+) and the Jsap1−/− ES cells (−/−) were grown in the presence of feeder cells with standard ES medium, and the number of ES cells was counted at given times. Three independent experiments with cultures initiated with 1 × 10⁵ cells/well in 6-well gelatin-coated plates were performed. The data are shown as the mean ± S.E. F, morphology and expression of cell surface stage-specific embryonic antigen-1 in the JSAP1-null ES cells. The parental CCE ES cells (+/+) , Jsap1+/− (+/−), and Jsap1−/− (−/−) were cultured in the presence of feeder cells with standard ES medium. Upper, transmission image. Lower, laser-scanning fluorescence microscopy for stage-specific embryonic antigen-1. Scale bar: 20 μm. G, expression of JSAP1 protein in wild type and Jsap1−/− ES cells. The parental CCE ES cells (+/+) , Jsap1+/− (+/−), and Jsap1−/− (−/−) were cultured in the absence of feeder cells with standard ES medium and subjected to laser-scanning fluorescence microscopy for JSAP1 (green) and nuclear DNA (TOPRO-3, blue). The merged images of each projection (13 sections) are shown, and the number of JSAP1-positive nuclei out of 300 independent nuclei is shown in parentheses. Scale bar: 15 μm.
FIG. 2. Embryoid body formation and neural differentiation. A, experimental schema of in vitro differentiation of ES cells. Undifferentiated ES cells were cultured in suspension for 4 days and formed small EBs (4\(^{-}\)). The EBs were further cultured for an additional 4 days in the presence (4\(^{+}\))/4\(^{-}\) or absence (4\(^{-}\)/4\(^{-}\)) of RA. Then the EBs were grown on gelatin-coated dishes for 7 days and differentiated into neurons and glial (4\(^{-}\)/4\(^{-}\)4\(^{-}\)/4\(^{-}\)) or various types of cells with endodermal, mesodermal, and ectodermal origins (4\(^{-}\)/4\(^{-}\)). B and C, embryoid body formation from the JSAP1-null ES cells. Parental CCE ES cells (Jsap1\(^{+/+}\)) and JSAP1-null ES cells (Jsap1\(^{-/-}\)) were subjected to 4\(^{-}\)/4\(^{-}\)/4\(^{-}\)/4\(^{-}\) protocols for EB formation. B, stereoscopic micrograph. Scale bar: 250 \(\mu\)m. C, histograms representing the size distribution of EBs. The area of a cross-section for each EB was determined using NIH image software, and the results are shown as histograms (n = 100). *, mean ± S.D.; a and b, p < 0.0001. D and E, the altered neurite outgrowth from the JSAP1-null embryoid bodies. The parental CCE ES cells (Jsap1\(^{+/+}\)) and JSAP1-null ES cells (Jsap1\(^{-/-}\)) were subjected to upper protocols for neuronal differentiation. D, immunofluorescent staining of the neurofilament. Scale bar: 100 \(\mu\)m. E, impaired neurite outgrowth from the JSAP1-null EBs. The neurite outgrowth was quantified by measuring the distance to the farthest end of the neurite from each EB. The distance was measured on a digital image of each EB using NIH image software, and the results are shown as histograms (n = 200). *, mean ± S.D.; a and b, p < 0.0001.
JNK3 and JSAP1 co-exist in a complex in vivo. We further attempted to detect JSAP1 in the immunocomplexes precipitated with anti-MEKK1, MLK3, MKK4, and MKK7 but failed to detect it (data not shown).

c-Jun and ATF2 were preferentially expressed in undifferentiated wild type ES cells, and the levels were significantly reduced in undifferentiated JSAP1-null ES cells (Fig. 3D, lanes 1). The expression level of Elk1 was not significantly altered in
JSAP1-null ES cells and EBs in comparison to wild type. The phosphorylation status of JNK and c-Jun was examined with anti-phospho-JNK (Thr-183 and Tyr-185) and anti-phospho-c-Jun (Ser-63) antibodies. Substantial levels of phosphorylated JNKs were detected in the early EBs (4/4), which were formed from wild type but not JSAP1-null mutants, and also in the differentiated cells from RA-treated JSAP1-null EBs as well as wild type EBs (Fig. 3B, lanes 2 and 4). Much lower levels of phosphorylated c-Jun were detected in the JSAP1-null ES cells or their early EBs than in wild type. In contrast, an increased level of phosphorylated c-Jun was detected in the JSAP1-null EBs (4/4), which were formed in the absence of RA, in comparison with the level seen in wild type EBs (Fig. 3B, lanes 5).

As shown in Fig. 3D, Jsap1, c-jun, Elk1, and Atf2 but not Jnk3 mRNAs were highly expressed in the undifferentiated wild type ES cells, and those mRNAs other than Jsap1 were also expressed in the JSAP1-null mutant. In the wild type a higher level of Jsap1 mRNA was expressed in the late EBs (4/4) and neurons differentiated from them as well as in undifferentiated ES cells (Fig. 3D, lanes 1, 3, and 4), in comparison with EBs and differentiated cells in the absence of RA (Fig. 3D, lanes 2, 5, and 6). The level of Jnk3 mRNA gradually increased throughout in vitro differentiation especially with RA treatment, both in wild type and JSAP1-null mutant. In contrast, the c-jun mRNA level apparently decreased in the early EBs (4) (Fig. 3D, lane 2) and then later recovered in both the wild type and JSAP1-null mutants. mRNAs for Elk1 and Atf2 were constantly expressed throughout the experiments in both the wild type and JSAP1-null mutants. Levels of Jnk3, c-jun, Elk1, and Atf2 mRNAs were not altered by loss of JSAP1.

Abnormal Ectoderm Formation in the JSAP1-null EBs—To examine the morphological alteration in EBs formed from wild type and JSAP1-null ES cells, sections prepared from EBs embedded in paraffin were processed for hematoxylin-eosin staining and indirect immunohistochemistry for JSAP1, JNK3, β-tubulin III, and Wnt1, as shown in Fig. 4. Seventy-six percent of JSAP1-null EBs (76/100 EBs) formed in the absence of RA (4/4) exhibited hyperplastic proliferation of ectodermal epithelium, thus forming a multitubular structure of varying sizes, whereas less than 5% of wild type EBs exhibited such a multitubular structure (Fig. 4, A1 and B1). JSAP1 mainly expressed in the ectoderm and outer layer of endoderm of wild type but not JSAP1-null EBs (Fig. 4, A2 and B2). JNK3 expression in wild type EBs was spatially similar to that of JSAP1, and a much lower expression of JNK3 was detected in the hyperplastic ectodermal layers of JSAP1-null EBs (Fig. 4, A3 and B3). A much higher level of β-tubulin III, a marker for immature neurons (25), was detected in the hyperplastic ectodermal layers of JSAP1-null EBs (Fig. 4, A3 and B3). JSAP1-null EBs formed in the presence of RA (4/4) were apparently smaller in size with a smaller cavity than wild type EBs (Fig. 4, C1 and D1). JNK3 as well as JSAP1 was highly expressed in the outer layer of endoderm and the ectodermal layer of the wild type (Fig. 4, C2 and C3), whereas JNK3 was expressed only in the outer layer of the endoderm of JSAP1-null EBs (Fig. 4, D3). Most ectodermal and endodermal cells in the wild type EBs exhibited a high level expression of β-tubulin III as well as Wnt1 (Fig. 4, C4 and C5), and both expressions were barely detectable in JSAP1-null EBs (Fig. 4, D4 and D5). These results correlated with the results from an RT-PCR analysis shown in Fig. 3A.

As shown in Fig. 4, E and F, only wild type but not JSAP1-null EBs (4/4) exhibited prominent terminal dUTP nick-end labeling-positive signals, especially in the cavity. A higher level of pro-caspase 3 and its processed forms (p24, p20, p17) was detected in the wild type but not JSAP1-null EBs (4/4) accompanied with enhanced cleavage of PARP (Fig. 3E). In JSAP1-null EBs, especially in the late phases (4/4, 4/4) with or without RA the expression level of PARP itself apparently decreased (Fig. 3E, lanes 3–6).

Expression of JSAP1 in the E7.5 and E12.5 Developing Mouse Embryos—The expression of JSAP1 in the developing mouse embryo was examined to evaluate its involvement in mouse embryogenesis. As shown in Fig. 5A, JSAP1 and JNK3 were simultaneously expressed in embryonic and extra-embryonic ectoderm and also in the extra-embryonic endoderm in E7.5 developing mouse embryos, whereas a high level of Wnt1 expression was seen only in the embryonic ectoderm. Lower but substantial levels of JSAP1, JNK3, and Wnt1 were also detected in the embryonic mesoderm. β-Tubulin III, JNK3, and Wnt1 were likely to be coexpressed with JSAP1, especially in the embryonic ectoderm, namely the neuroectoderm, from where developmental neurogenesis occurs (40, 41) (Fig. 5B). A Western blotting analysis of the whole embryo also demonstrated a high level expression of JSAP1 (p180) and JNK3 in the embryos (E6.5 and E7.5) (Fig. 5C).

A high level of JSAP1 expression was further detected at the midbrain-hindbrain junction in E12.5 embryos, and where Pax2 and Wnt1, markers for definition of the midbrain-hindbrain junction, were coexpressed (42). The expression of JSAP1 was also detected in the ependymal layer of the forebrain and spinal cord.

Expression of JSAP1 in the Neurons Differentiated from Wild Type EBs and Altered Neuronal Differentiation from JSAP1-null EBs—In the wild type neurons differentiated from EBs formed in the presence of RA (4/4), JSAP1 was localized in the neurites, growth cones, and to some extent in the soma of neurons (Fig. 6, A and B; Fig. 7, A–C). GFAP-positive glia-like cells which express JSAP1 (Fig. 6C) were also efficiently differentiated from wild type EBs but much less efficiently from JSAP1-null EBs (Fig. 6D). About 10–20% of neurofilament-positive neurites were tyrosine hydroxylase-positive in the neurons differentiated from both wild type and JSAP1-null EBs treated with RA, although neurofilament-positive neurites were poorly elongated from JSAP1-null EBs in comparison with wild type (Fig. 6E). On the other hand JSAP1-null EBs, which were formed in the absence of RA (4/4), elongated neurites expressing neurofilament, GAP43, and tyrosine hydroxylase or differentiated into GFAP-positive glia-like cells more efficiently than did wild type EBs (Fig. 6, F and G).

In JSAP1-null Neurons, Synaptophysin Accumulated in the Soma—In the wild type neurons differentiated from RA-treated EBs, synaptophysin as well as JSAP1 was detected evenly from the soma to the neurite, thus indicating that the synaptic vesicles were functionally transported from the soma to the terminal of each neurite (Fig. 7, A–D). On the other hand, in JSAP1-null mutant neurons, a much stronger signal for synaptophysin was detected in the soma than in neurites (Fig. 7, E and F). To elucidate any quantitative differences in the synaptophysin distribution between the wild type and mutant neurons, fluorescence intensity was digitized using NIH image software (Fig. 7G). The accumulation of synaptophysin in the soma of the JSAP1-null mutant neurons was more than three times greater than that observed in the soma of wild type neurons.
DISCUSSION

In the present study we obtained evidence that JSAP1-null ES cells exhibit large alterations in levels and/or functions of multiple components of JNK-signaling cascades, resulting in substantial alterations in the process of differentiation of cells within EBs; however, our results should be replicated with mouse embryos generated from the JSAP1-null ES cells. The JSAP1 mutation appears to have strong effects on the size of EBs, suggesting the possibility that multiple changes in ES cell differentiation observed in the JSAP1-null ES cells may result as an indirect effect of the altered EB size. Because we observed altered expression of various components of JNK cascades even in undifferentiated JSAP1-null ES cells and because an altered expression pattern of various differentiation markers during formation of early EB (4+/−) from the JSAP1-null ES cells was evident before the effects on the size of EB became apparent,
we concluded that most of the alterations observed in EBs derived from the JSAP1-null ES cells were not due to an indirect effect of the size, thus delineating the roles of JSAP1 during the early embryogenesis.

Expression of JSAP1 during in Vitro Differentiation of ES Cells and Early Embryogenesis—The Jsap1 mRNA level was substantially high in the undifferentiated ES cells and was slightly altered during EB formation but apparently decreased in the differentiated cells from EBs that were formed in the absence of RA, thus suggesting that the transcription of the Jsap1 gene may be partly regulated during cell differentiation. Furthermore, the JSAP1 protein levels were more dramatically altered during EB formation, thus suggesting that the stability of JSAP1 protein is also regulated during cell differentiation as well. In the RA-treated EBs and neurons differentiated from them, expression of two or three polypeptides (p190/p180 and p150) reactive with anti-JSAP1 were strongly induced, and only the p180 was detected in undifferentiated ES cells or EBs formed in the absence of RA. Multiple polypeptides of JSAP1 detected by Western blotting may represent variants encoded by alternatively spliced forms of JSAP1 transcripts (43) or those modified in a post-translational manner (15). Accordingly, we detected several splicing variants of Jsap1 mRNA during the in vitro differentiation.2 It is noteworthy that only the p190 JSAP1 was co-precipitated with anti-JNK3 from RA-treated EBs, thus indicating that alternative splicing or post-translational modification of JSAP1 may regulate its interaction with JNKs.

The expression of JSAP1 in the RA-treated EBs (4/4') was very high and mostly detected in the β-tubulin III-positive neuroectoderm and where JNK3 and Wnt1 were also coexpressed. In developing mouse embryos the neuroectoderm formation initiates during stages E7.0–7.5 (40) and expression levels of JSAP1, JNK3, and Wnt1 were as high in the β-tubulin III-positive neuroectoderm of the E7.5 embryo as in the RA-treated EBs (4/4'), thus indicating that the neuroectoderm formed in RA-treated EBs (4/4') may represent the neuroectoderm in E7.5 mouse embryos. Furthermore, a high level of JASP1 expression was detected at the midbrain-hindbrain junction of E12.5 embryo with coexpression of Pax2 and Wnt1, which are considered as important factors regulating isthmic organization of the midbrain-hindbrain junction (42, 44), thus suggesting that JSAP1 may contribute to the formation of central nervous system as well as the early embryonic neurogenesis.

JSAP1 Negatively Regulates Growth of Undifferentiated ES Cells—JSAP1-null ES cells apparently grew faster than did parental CCE cells, probably reflecting the loss of either JNK1/2-c-Jun- or JNK1/2-ATF2-signaling cascades. It has been established that unphosphorylated c-Jun and ATF2 are subjected to proteolysis by proteasomes through ubiquitinylation (45, 46). In our results the level of phosphorylated c-Jun decreased in JSAP1-null ES cells in undifferentiated ES cells. We hypothesize that a loss of functional JSAP1 results in the destabilization of JNKs, and their substrates, such as c-Jun and ATF2, remain unphosphorylated and then are degraded. It is, thus, likely that JSAP1 scaffold protein is required to maintain the stability of components for particular JNK signaling modules, at least in the undifferentiated ES cells. We could not rule out the possibility that the accelerated proliferation of the JSAP1-null ES cells may partly be attributed to an extracellular signal-regulated kinase cascade, since JSAP1 has been shown to have a capacity to suppress the extracellular signal-regulated kinase-signaling pathway through its interaction with MEK1 and Raf-1 (47), and the expression level of Elk1, which is a main target of extracellular signal-regulated kinase 1/2, in undifferentiated JSAP1-null ES cells was apparently higher than that in the wild type ES cells.

JSAP1 Negatively Regulate Neural Fate Specification of ES Cells in the Absence of RA—EBs formed from JSAP1-null ES cells in the absence of RA exhibited hyperplastic proliferation of ectodermal epithelium with a particular multitudinal struc-

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a P. Xu and Y. Nakabeppu, unpublished results.
ture accompanied with an increased expression of neural specific genes, such as *Emx2*, *Otx1*, *Pax5*, and *Wnt1*, and resulted in a more efficient differentiation of neurons and glia in comparison with wild type EBs.

In vertebrates the patterning of neuroectoderm along the anteroposterior axis is initiated during gastrulation and mainly regulated by Wnts and fibroblast growth factor signal pathways (44, 48). It has been established that an appropriate level of Wnt activity may specify posterior-to-anterior fates within the neural plate that arise from the neuroectoderm during the head formation (49, 50). *Wnt1* knockout mice have a severe phenotype of most loss of the midbrain and cerebellum (51). However, in mice lacking the *Six3* gene, which is essential for forebrain development, the prosencephalon was severely truncated, and expression of *Wnt1* was rostrally expanded, thus suggesting that the suppression of Wnt signal is essential.
JSAP1 also resulted in an up-regulation of also causes severe abnormalities during pattern formation in provide an appropriate negative signaling for the loop. feedback loop with fibroblast growth factor 8 (54), JSAP1 may as well as Pax5 sin in neurons differentiated from wild type EB. JSAP1 (52). These facts strongly indicate that an increased expression abnormal hyperplastic proliferation of the neuroectoderm. of Wnt1 in the JSAP1-null EBs may be the cause of their (52). In the JSAP1-null EBs (4–4/1), the stabilization JSAP1 Positively Regulates Apoptosis in Association with JNK3 during Neuroectoderm Formation Induced by RA—RA significantly increased the expression of both JSAP1 and JNK3 in wild type but not JSAP1-null EBs, and apoptosis was highly induced only in the former along with processing of caspase 3 and PARP. The JNK3 has been reported to positively regulate apoptosis especially in neurons (55). Interaction of JSAP1 with JNK3 in the 4–4/1 EBs indicates that JSAP1 may positively regulate JNK3 function or its stability as a scaffold protein for the JNK cascade, as proposed previously (15–17), at least during neuroectoderm formation in the EBs, thus inducing a prominent degree of apoptosis through the caspase 3 pathway. We may suggest that JSAP1 plays an important role in association with JNK3 during neuroectoderm formation in the mouse embryo, since JSAP1 and JNK3 are coexpressed in the β-tubulin III-positive neuroectoderm of E7.5 embryo. Jnk1/ Jnk3 and Jnk2/ Jnk3 double knockout but not Jnk1/ Jnk2 double knockout mice develop normally; therefore, it is likely that JNK1 and JNK2, which mainly regulate apoptosis at midgestation during embryogenesis (5), can compensate for any JNK3 deficiency during neuroectoderm formation.

Functional Roles of JSAP1 in Regulation of Vesicle Transport in Neurons—From the JSAP1-null EBs formed in the presence of RA, the apparent outgrowth of neurites was poor, thus suggesting that JSAP1 is also involved in the late stage of neural maturation. In Drosophila, a JSAP1 homolog has been identified as Sunday Driver (SYD), which mediates kinesin-dependent axonal transport (56), and UNC-16, a JSAP1 homolog in Caenorhabditis elegans was reported to regulate the localization of the vesicular cargo by integrating JNK signaling and kinesin-1 transport (57).

In the JSAP1-null neurons, synaptophysin, which is a component of the synaptic vesicle and is usually localized in axon (58), was mostly localized in the soma and much less in neurites, thus suggesting that a loss of JSAP1 partly retarded the axonal transport in mouse neurones, thus resulting in a poor outgrowth of neurites. In the wild type neurons, JSAP1 was also detected in the terminal of the neurites, namely growth cone, as previously reported (16, 59). Our results, thus, also provide evidence that JSAP1, a mammalian scaffold protein for JNKs, is involved in the regulation of vesicle transport, as are SYD and UNC-16.

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