

Regulation of N-cadherin-based cell-cell interaction by JSAP1

メタデータ	言語: eng 出版者: 公開日: 2017-10-05 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	http://hdl.handle.net/2297/3640



Regulation of N-cadherin-based cell–cell interaction by JSAP1 scaffold in PC12h cells

Munkhuu Bayarsaikhan^a, Takahisa Takino^b, Davaakhuu Gantulga^a,
Hiroshi Sato^b, Takashi Ito^c, Katsuji Yoshioka^{a,*}

^a Division of Molecular Cell Signaling, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa 920-0934, Japan

^b Division of Molecular Virology and Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa 920-0934, Japan

^c Department of Computational Biology, Graduate School of Frontier Sciences, University of Tokyo, Kashiwa 277-8561, Japan

Received 30 November 2006

Abstract

We previously reported that the level of c-Jun NH₂-terminal kinase (JNK)/stress-activated protein kinase-associated protein 1 (JSAP1), a scaffold protein for JNK signaling, increases dramatically during nerve growth factor (NGF)-induced differentiation of PC12h cells. In the present study, we investigated the function of JSAP1 during PC12h cell differentiation by knocking down the level of JSAP1. The depletion of JSAP1 caused NGF-treated PC12h cells to form aggregates and impaired their differentiation. The aggregation was not observed in JSAP1-depleted cells that were untreated or treated with epidermal growth factor. Immunocytochemical studies indicated that N-cadherin, but not E-cadherin, was localized to sites of cell–cell contact in the aggregated cells. Furthermore, an inhibitory anti-N-cadherin antibody completely blocked the aggregation. Taken together, these results suggest that JSAP1 regulates cell–cell interactions in PC12h cells specifically in the NGF-induced signaling pathway, and does so by modulating N-cadherin.

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Keywords: Cadherin; Differentiation; JNK; MAP kinase; NGF; RNA interference; Scaffold protein

Mammalian mitogen-activated protein kinases (MAPKs), including the extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK), and p38 subgroups, are activated in response to a variety of extracellular and intracellular stimuli [1,2]. Mammalian MAPK signaling pathways, in which MAPK is activated by a phosphorylation relay, from MAPK kinase kinase to MAPK kinase to MAPK, play pivotal roles in multiple cellular processes, including proliferation, differentiation, apoptosis, and migration [1–4]. Scaffold proteins of the mammalian MAPK cascades are thought to function in the spatio-temporal regulation of these pathways by organizing the MAPK signaling components into functional modules [5,6]. The functional complexes enable the efficient

signal transduction of specific MAPK cascades. JNK/stress-activated protein kinase-associated protein 1 (JSAP1, also known as JNK-interacting protein 3) was initially identified as a JNK-binding protein by our group and others, and biochemical studies have suggested that JSAP1 functions as a scaffold protein for JNK MAPK cascades [7–9]. Targeted gene-disruption studies in mice and an in vitro differentiation study with *jsap1*-deficient mouse ES cells showed that JSAP1 is required for embryonic neurogenesis [10–12].

The expression level of JSAP1 in rat pheochromocytoma PC12 cells increases dramatically during their nerve growth factor (NGF)-induced differentiation into neuron-like cells [8,13]. However, the physiological function of JSAP1 remains to be elucidated. In the present study, we established PC12 cell lines expressing a short hairpin RNA (shRNA) against JSAP1, in which JSAP1 expression is severely decreased, and we used these JSAP1 knockdown

* Corresponding author. Fax: +81 76 234 4532.

E-mail address: katsuji@kenroku.kanazawa-u.ac.jp (K. Yoshioka).

(KD) cells to investigate the function of JSAP1. Our results suggest that JSAP1 regulates cell–cell interactions in PC12 cells specifically in the NGF-induced signaling pathway, by modulating N-cadherin.

Materials and methods

Cell culture and reagents. PC12h cells, a subclone of PC12 cells [14], were cultured on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich) containing 5% fetal bovine serum (FBS) and 5% horse serum. For differentiation, PC12h cells were cultured in DMEM containing 5% FBS and 5% horse serum at a density of 0.5 to 1×10^4 cells/cm² overnight on collagen-coated dishes. The medium was then changed to DMEM containing 50 ng/ml NGF (2.5S form, Alomone Labs) and 1% horse serum. Madin-Darby canine kidney (MDCK) and human embryo kidney (HEK) 293T cells were cultured in DMEM containing 10% FBS. Type I collagen and Hoechst 33258 were purchased from Wako Pure Chemical Industries and Nacalai Tesque, respectively. Epidermal growth factor (EGF), puromycin, and Sequa-brene were from Sigma–Aldrich.

Antibodies. Monoclonal antibodies against E-cadherin, N-cadherin, β -catenin, and p120-catenin were purchased from BD Biosciences. An affinity-purified anti-JSAP1 polyclonal antibody was described previously [15,16]. A rabbit anti-JNK-associated leucine zipper protein (JLP) antibody was raised against a peptide (CTLSQLPGDKSKAFDF) corresponding to amino acid residues 581–595 of mouse JLP (GenBank Accession No. AF327451), and was affinity purified using the peptide. The anti-actin polyclonal antibody and anti-N-cadherin monoclonal antibody, which reacts with the N-terminal half of the extracellular domain of N-cadherin and inhibits adherens junction formation, were obtained from Sigma–Aldrich. The anti-JNK polyclonal antibody and anti-phospho-specific JNK monoclonal antibody were from Cell Signaling.

Plasmid construction. The retroviral vector pMKO.1-puro [17] was kindly provided by Dr. W.C. Hahn (Dana-Farber Cancer Institute, Harvard Medical School). The human U6 promoter in the pMKO.1-puro was replaced with the mouse U6 promoter by introducing a 0.3-kb *Bam*HI–*Age*I fragment containing the mouse U6 promoter, which was obtained by PCR using the vector pBAsi-mU6 (TaKaRa), into *Bg*II/*Age*I-digested pMKO.1-puro, to generate pmMKO.1-puro. The following phosphorylated and annealed small interfering RNA oligos with hairpin sequences targeted against JSAP1 were inserted into the *Age*I and *Eco*RI sites of pmMKO.1-puro: JSAP1-shRNA1, forward, 5'-CCGGCAGTGTACACAAGAACAACAACCTCGAGTTGTTGTTCTTTGTGACACTGTTTTG-3' and reverse, 5'-AATTCAAAAA CAGTGTACACAAGAACACAATTTGTTGTTCTTTGTGACACTG-3'; JSAP1-shRNA2, forward, CCGGCCGAATCAAAGAGCTTGAAGCCTCGAGTCTTC AAGCTCTTTGATTTCGGTTTTTG-3' and reverse, 5'-AATTCAAAAA ACCGAATCAAAGAGCTTGAAGACTCGAGTCTTCAAGCTCTTT GATTTCGG-3'; JSAP1-shRNA3, forward, 5'-CCG GCTCAC AGAGC ATGTCTTTACTCTCGAGAGTAAAGACATGCTCTGTGAGTTTT TG-3' and reverse, 5'-AATTCAAAAACTCACAGAGCATGTCTTT ACTCTCGAGAGTAAAGACATGCTCTGTGAG-3'; JSAP1-shRNA4, forward, 5'-CCGGCGGTAGCCAACTGGAAGAAGTCTCGAGAC TTCTTCCAGTTGGCTACCGTTTTTG-3' and reverse, 5'-AATTCA AAAACGGTAGCCAACTGGAAGAAGTCTCGAGACTTCTTCCA GTTGGCTACCG-3', where the underlined letters represent mouse JSAP1 sequences (GenBank Accession No. AB005662). The resultant vectors were named pmMKO.1-puro-JSAP1-shRNA1–4.

Western blotting. Cultured cells were lysed in the following buffer: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% SDS, and protease inhibitor cocktail (Sigma–Aldrich). The extracts were spun for 10 min at 12,000g at 4 °C, and the supernatants were used as the protein samples. Western blot analysis was carried out as described previously [13].

Virus production and screening of cell lines stably expressing JSAP1 shRNA. HEK 293T cells were plated on 60-mm dishes at a density of 2×10^6 cells/dish. After 24 h, the cells were co-transfected with 1 μ g of

empty pmMKO.1-puro, pmMKO.1-puro-JSAP1-shRNA1, -shRNA2, -shRNA3, or -shRNA4 and 1 μ g of pCL-Ampho (Imgenex), a vector encoding a replication-defective helper virus, using FuGENE 6 (Roche Diagnostics). Forty-eight hours after transfection, the supernatants, containing amphotropic retroviruses, were collected, passed through a filter (0.45 μ m), supplemented with Sequa-brene (final concentration, 8 μ g/ml), and used for infection. PC12h cells were plated on collagen-coated dishes (1×10^6 cells/35-mm dish). After 24 h, the cells were infected with the amphotropic retroviruses, and another 24 h later, selection of the infected cells was started in medium containing 500 ng/ml puromycin. The selected cell populations were subjected to Western blotting with an anti-JSAP1 antibody to examine the silencing efficiency of the JSAP1-shRNAs. To obtain clones expressing JSAP1-shRNA1, PC12h cells plated on collagen-coated dishes (1×10^6 cells/35-mm dish) were infected with serially diluted recombinant retrovirus for JSAP1-shRNA1, and the cells were selected as described above. The expression of JSAP1 protein in independent clones was examined by Western blotting using the anti-JSAP1 antibody.

Time-lapse video microscopy. Cell movements were monitored using an Olympus inverted microscope as described previously [18]. Video images were collected at one-hour intervals for 48 h, and the image stacks were converted to QuickTime movies.

Immunofluorescence. PC12h cells were washed once with phosphate-buffered-saline (PBS) and fixed in 4% paraformaldehyde for 15 min. They were then permeabilized by incubation in PBS containing 0.1% Triton X-100 for 20 min on ice, blocked in PBS containing 0.1% Tween 20 (PBST) and 3% bovine serum albumin for 1 h at room temperature, and then incubated overnight at 4 °C with primary antibodies. After being washed with PBST, the cells were incubated for 1 h with an Alexa 546-conjugated secondary antibody (Molecular Probes). The stained cells were then examined by fluorescence microscopy.

Results

Screening for an effective shRNA against JSAP1 and establishment of stable PC12h cell lines expressing the shRNA

To investigate the physiological functions of JSAP1, we used the RNA interference (RNAi) approach. We examined the effectiveness of four JSAP1-shRNAs: JSAP1-shRNA1, -shRNA2, -shRNA3, and -shRNA4 (see Materials and methods). To this end, PC12h cells were infected with the respective recombinant retroviruses for these JSAP1-shRNAs, selected in the presence of puromycin, and analyzed by Western blotting. The expression level of JSAP1 protein in the JSAP1-shRNA4-expressing cells was comparable to that in the wild-type parent PC12h cells and in control PC12h (PC12h^{ctrl}) cells that had been infected with empty retrovirus (Fig. 1A; compare lanes 1, 2, and 6). The other three JSAP1-shRNAs decreased the JSAP1 expression in PC12h cells, with the most effective shRNA being JSAP1-shRNA1 (Fig. 1A). None of these JSAP1-shRNAs decreased the level of JLP, a highly related family member of JSAP1 [19] (Fig. 1A). In PC12h cells treated with NGF for 5 days, JSAP1-shRNA1 reduced the JSAP1 expression by 70–80% (Fig. 1B). Because the puromycin-resistant cells infected with the JSAP1-shRNA1 expression retrovirus could be heterogeneous with respect to JSAP1 expression, and furthermore, because cells expressing somewhat low or even quite low levels of JSAP1 protein might behave the same as wild-type PC12h cells, we

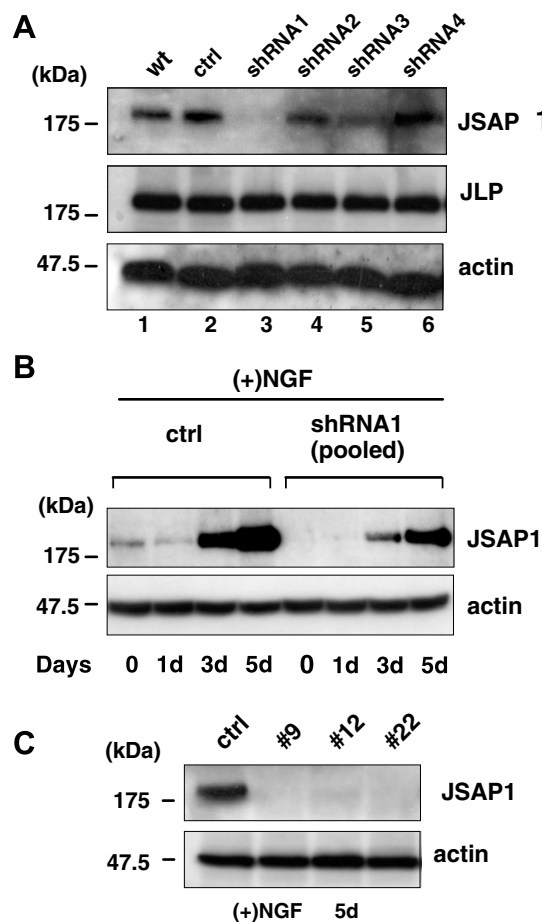


Fig. 1. Establishment of stable PC12h cell lines expressing shRNA against JSAP1. (A) PC12h cells were infected independently with recombinant retroviruses for JSAP1-shRNA1, -shRNA2, -shRNA3, and -shRNA4, and selected in the presence of puromycin. Cells were cultured without NGF. Total cell lysates were prepared from the respective pooled cells, and subjected to Western blotting with antibodies against JSAP1 and JLP, a JSAP1 family member. The wild-type (wt) parent PC12h cells, and the control (ctrl) PC12h cells, which were infected with empty retrovirus, were used as controls. (B) The control PC12h cells and the pooled PC12h cells stably expressing JSAP1-shRNA1 were treated with NGF (50 ng/ml) for the indicated periods: 1d, 1 day; 3d, 3 days; 5d, 5 days. JSAP1 protein expression was analyzed as in (A). (C) The control PC12h cells and the JSAP1 KD cells of clones #9, #12, and #22, which were obtained after screening the pooled cells infected with JSAP1-shRNA1-expressing retrovirus, were cultured for 5 days in the presence of NGF (50 ng/ml), and subjected to Western blotting as in (A). The expression of actin is shown as a loading control. The positions of protein size markers are indicated on the left.

screened for independent clones that barely expressed any JSAP1 protein. Indeed, the expression level of JSAP1 protein was different among distinct clones (data not shown), and almost no expression of JSAP1 protein was observed in clones #9, #12, and #22 after 5 days of NGF treatment (Fig. 1C).

JSAP1 KD PC12h cells form aggregates in response to NGF

The PC12h^{ctrl} cells and the JSAP1 KD cells of clones #9, #12, and #22 were cultured in the presence of NGF for 3

days, and then examined for morphological differences. Most of the PC12h^{ctrl} cells extended neurites during the process of NGF-induced differentiation (Fig. 2A(b)), consistent with our previous observations [13]. In contrast, the depletion of JSAP1 caused NGF-treated PC12h cells to form aggregates and impaired their differentiation. In the aggregates, some of the peripheral cells possessed neurites, but the remaining peripheral cells and the cells inside the aggregates did not (Fig. 2A(d, f, and h)). The average number of cells per cell unit, where either a single cell or a cell cluster of two or more cells was considered one cell unit, was 1.6 for the PC12h^{ctrl} cells, and 7.2, 5.4, and 4.5 for clones #9, #12, and #22, respectively (Fig. 2B). The corresponding number in the pooled PC12h cell population expressing JSAP1-shRNA1 was 2.8, which was smaller than the numbers obtained for the JSAP1 KD clones, as expected (Fig. 2B). Note that in the PC12h^{ctrl} cells, the average cell number per cell unit decreased after treatment with NGF (Fig. 2B). Whether cultured with EGF or without a growth factor, the cells of the JSAP1 KD clones showed no apparent difference in aggregation from the PC12h^{ctrl} cells (Fig. 2A, left panels; data not shown). Indeed, the average cell numbers per cell unit were similar among the PC12h^{ctrl} cells and JSAP1 KD clones in the absence of NGF (Fig. 2B). These observations indicate that the phenomenon of cell–cell aggregation occurs specifically as a result of NGF-induced signaling in PC12h cells. To clarify the molecular mechanism underlying the formation of JSAP1 KD cellular aggregates, we performed further experiments using clones #9 and #22, with the PC12h^{ctrl} cells as a control.

JSAP1 KD causes cell aggregation by promoting N-cadherin-mediated cell–cell adhesion in NGF-treated PC12h cells

We first examined cell behaviors, focusing on cell–cell association and dissociation, by time-lapse video microscopy. After NGF treatment, video images were collected for 48 h at one-hour intervals, and the images were converted into movies. The PC12h^{ctrl} cells seemed to undergo active cell–cell dissociation and very weak cell–cell association, whereas the opposite cell behaviors were exhibited by the JSAP1 KD cells (Fig. 3A). These results suggested that a cell–cell adhesion molecule(s) might be involved in the formation of the aggregates. To examine this possibility, we performed immunostaining for the classical cadherins, the E- and N-cadherins. As shown in Fig. 3B, N-cadherin, but not E-cadherin, was detected at almost all the sites of cell–cell contact in the JSAP1 KD cellular aggregates. We confirmed the ability of the anti-E-cadherin antibody to detect its protein at cell–cell contacts using the MDCK epithelial cell line (data not shown). We next asked whether N-cadherin is the major molecule promoting cell–cell adhesion in the aggregates. When cultured in the presence of an inhibitory antibody for N-cadherin, no aggregates were seen in the NGF-treated JSAP1 KD cells (Fig. 3C). The

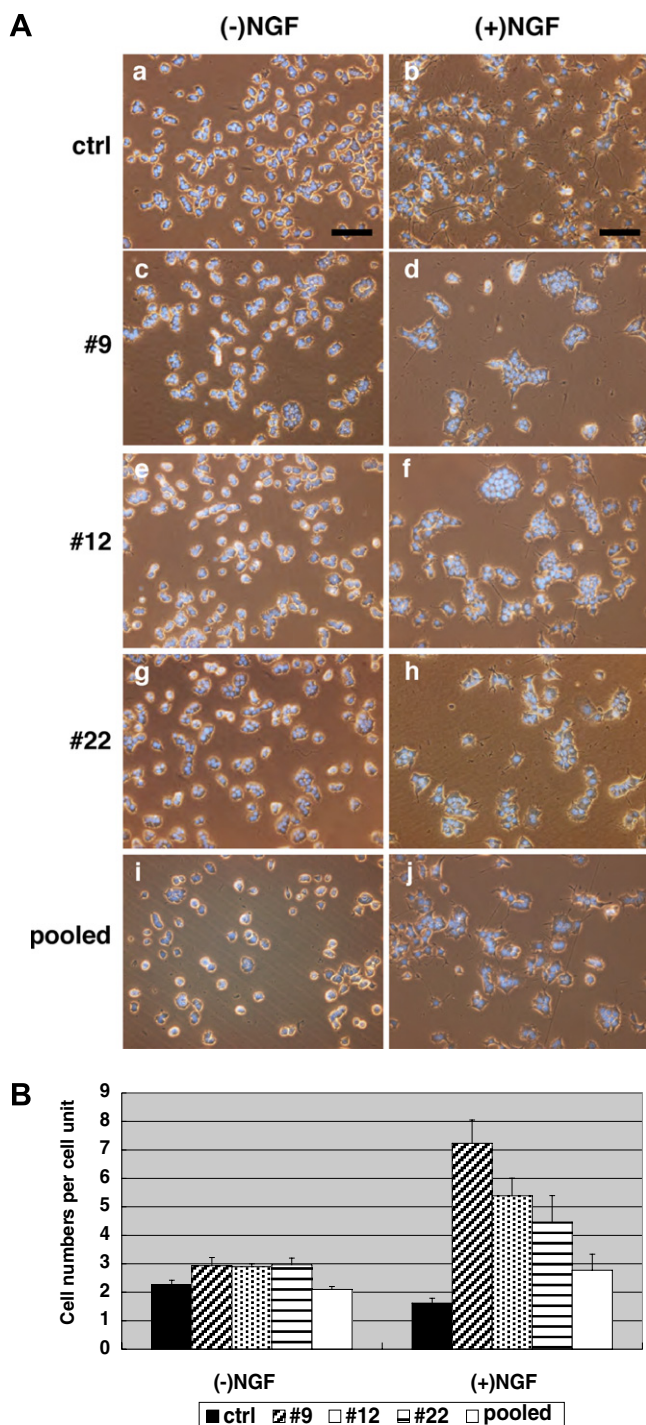


Fig. 2. NGF-induced cell–cell aggregation in JSAP1 KD PC12h cells. (A) The control (ctrl) PC12h cells (a,b), JSAP1 KD cells from clones #9 (c,d), #12 (e,f), and #22 (g,h), and pooled cells (i,j) expressing JSAP1-shRNA1 were cultured on collagen-coated dishes for 3 days in the presence or absence of NGF (50 ng/ml), and stained with Hoechst 33258. Scale bar, 100 μ m. (B) To quantify the results of (A), the average number of cells per cell unit, which consisted of a single cell or a cluster of two or more cells, was determined. Over 100 cells were examined in each case. Values are the means \pm SEM from three independent experiments.

inhibitory antibody, however, did not affect the cell–cell adhesion of the MDCK cells under similar culture conditions (data not shown). We also analyzed the expression

levels of the E- and N-cadherins, and their related proteins, β -catenin and p120-catenin, by Western blotting, using cells harvested 0, 1, 3, and 5 days after NGF treatment. The expression levels of the E- and N-cadherins increased slightly in the JSAP1 KD cells compared with the PC12h^{ctrl} cells, in response to NGF stimulation (Fig. 3D), and there were no significant differences in the levels of β -catenin and p120-catenin between the PC12h^{ctrl} cells and JSAP1 KD clones #9 and #22 (data not shown). Taken together, these results suggested that during NGF-induced signaling in PC12h cells, JSAP1 regulates the expression of N-cadherin, and that the depletion of JSAP1 disrupts the normal regulation of N-cadherin-based cell–cell adhesion, leading to the formation of cellular aggregates.

Immunoblotting analysis of JNK in the NGF-treated control and JSAP1 KD PC12h cells

Because JSAP1 has been identified as a scaffold protein for JNK signaling [7,8], we asked whether the depletion of JSAP1 affects the activation of JNK during NGF-induced signaling in PC12h cells. For this purpose, we examined the expression of the phosphorylated, activated form of JNK by Western blotting, using cells harvested 0, 1, 3, and 5 days after NGF treatment (Fig. 4). The total JNK levels were similar between the PC12h^{ctrl} and the JSAP1 KD cells of clones #9 and #22, and the level of phosphorylated and activated JNK increased greatly in the PC12h^{ctrl} cells on days 3 and 5; activated JNK levels also increased, but to a lesser degree, in the JSAP1 KD cells.

Discussion

Cell–cell interactions and other cellular processes, such as cell proliferation, differentiation, apoptosis, and migration, are coordinately regulated in multicellular organisms during development and the subsequent maintenance of homeostasis. Here we investigated the function of JSAP1, a scaffold protein for JNK MAPK cascades, during NGF-induced differentiation, using the rat pheochromocytoma cell line PC12h as a model system. We first established stable cell lines expressing an shRNA against JSAP1, in which there was almost no expression of JSAP1 protein (Fig. 1C), and found that the depletion of JSAP1 caused NGF-treated PC12h cells to aggregate and impaired their differentiation (Fig. 2). These phenomena were not observed in JSAP1 KD cells that were untreated or treated with EGF, indicating that the depletion of JSAP1 causes these defects in PC12h cells specifically during NGF-induced signaling.

Immunostaining analysis showed the presence of N-cadherin, but not E-cadherin, at the cell–cell contact sites of the aggregated JSAP1-depleted cells (Fig. 3B). Furthermore, an anti-N-cadherin antibody that reacts with the extracellular domain of N-cadherin and is known to inhibit adherens junction formation, completely blocked the aggregation (Fig. 3C). In addition, the expression level of

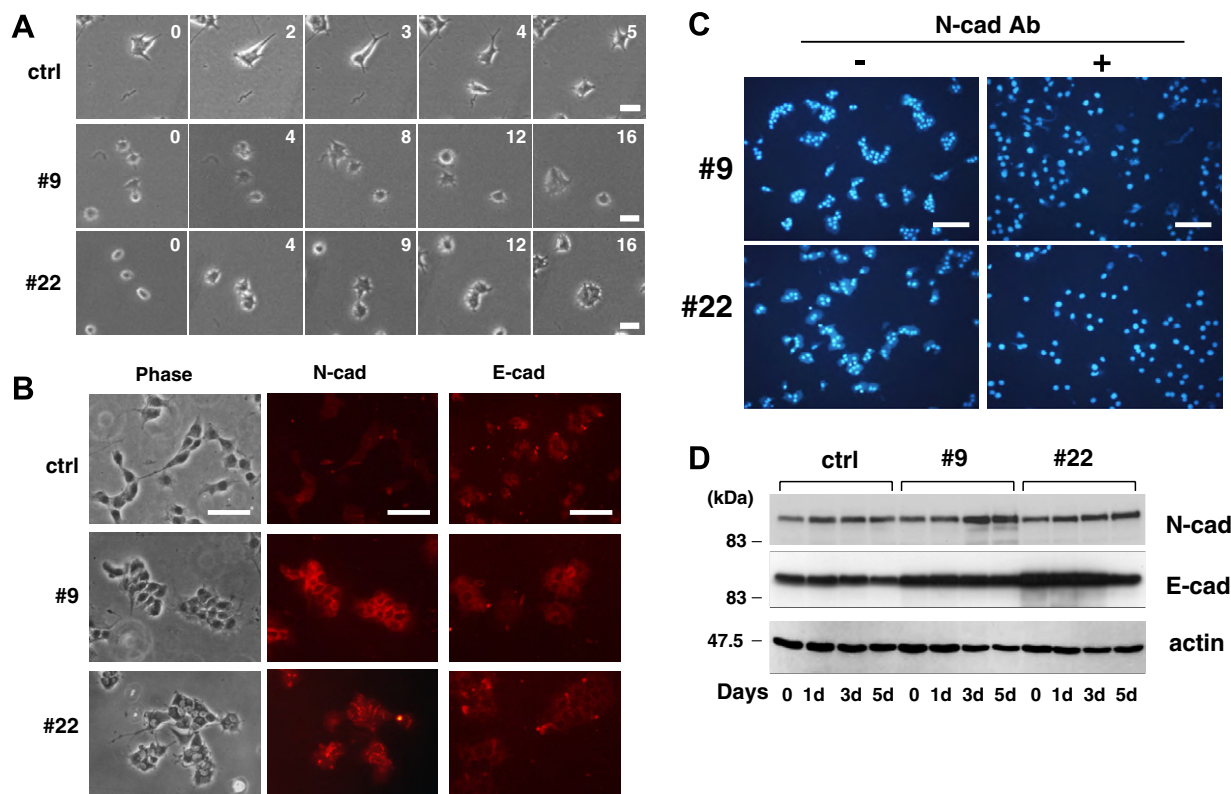


Fig. 3. N-cadherin-mediated cell-cell adhesion in NGF-treated JSAP1 KD cells. (A) The control (ctrl) PC12h cells and the JSAP1 KD cells from clones #9 and #22 were cultured on collagen-coated dishes and monitored by time-lapse video microscopy at one-hour intervals for 48 h, in medium containing NGF (50 ng/ml). Representative video images of the control cells and the JSAP1 KD cells from clones #9 and #22 are shown. Numbers represent the time in hours after the addition of NGF. Scale bar, 25 μ m. (B) The control (ctrl) PC12h cells and the JSAP1 KD cells of clones #9 and #22 were cultured on collagen-coated dishes for 3 days in the presence of NGF (50 ng/ml). The cells were then fixed and immunostained for E- and N-cadherin. Scale bar, 50 μ m. (C) The JSAP1 KD cells of clones #9 and #22 were cultured on collagen-coated dishes for 3 days in the presence of NGF (50 ng/ml) with or without an inhibitory antibody for N-cadherin. The final concentration of the inhibitory anti-N-cadherin antibody was 300 μ g/ml. The cells were then stained with Hoechst 33258. Scale bar, 100 μ m. (D) Control (ctrl) PC12h cells and JSAP1 KD cells from clones #9 and #22 were cultured on collagen-coated dishes for 1–5 days in the presence of NGF (50 ng/ml), as indicated: 1d, 1 day; 3d, 3 days; 5d, 5 days. Total cell lysates were prepared, and analyzed by Western blotting with anti-N-cadherin or anti-E-cadherin antibodies (upper two panels). Actin expression is shown as a loading control (bottom panel). The positions of protein size markers are indicated on the left. E-cad, E-cadherin; N-cad, N-cadherin; Ab, antibody.

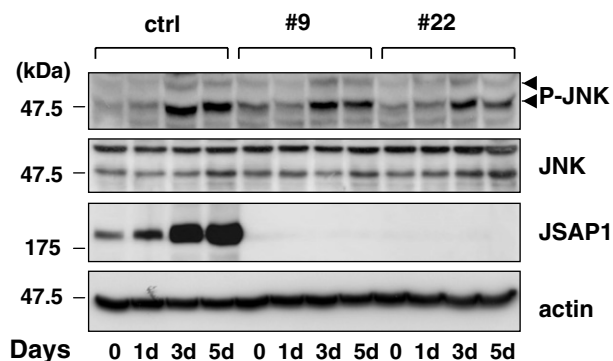


Fig. 4. Activation of JNK and expression of JSAP1 during NGF-induced signaling in the control and JSAP1 KD PC12h cells. The control (ctrl) PC12h cells and the JSAP1 KD cells from clones #9 and #22 were cultured on collagen-coated dishes for 1–5 days in the presence of NGF (50 ng/ml), as indicated: 1d, 1 day; 3d, 3 days; 5d, 5 days. Total cell lysates were then prepared and analyzed by Western blotting with anti-phospho-JNK (P-JNK), anti-JNK, and anti-JSAP1 antibodies (upper three panels). Arrowheads indicate the positions of phosphorylated JNKs. The expression of actin is shown as a loading control (bottom panel). The positions of protein size markers are indicated on the left.

N-cadherin increased slightly in the JSAP1 KD cells compared with the PC12h^{ctrl} cells, in response to NGF stimulation, (Fig. 3D). These results strongly suggest that the key binding molecule at the cell-cell junctions is N-cadherin, and that JSAP1 regulates the cell-cell interaction in NGF-induced PC12h cells by modulating N-cadherin expression. Chen et al. [20] reported that the overexpression of modifier of cell adhesion [MOCA; also known as dedicator of cytokinesis 3 (DOCK3)] in HEK293T and PC12 cells, among others, promoted N-cadherin-mediated cell-cell adhesion in association with increased N-cadherin expression. This phenotype is similar to that observed in our JSAP1 KD cells. Thus, JSAP1 might regulate the expression of N-cadherin by modulating the expression of MOCA and/or other proteins with a similar function. However, we cannot rule out the possibility that JSAP1 modulates a different aspect of N-cadherin function. It is possible that JSAP1 regulates the subcellular localization of N-cadherin, which is a transmembrane protein, by modulating the trafficking of N-cadherin to and/or from the cell

surface by exocytic and/or endocytic pathways. The altered subcellular localization of N-cadherin in the JSAP1 KD PC12h cells might interfere with cell–cell dissociation and enhance cell–cell association, which together could promote cell aggregation.

In the present study, we demonstrated that the scaffold protein JSAP1 regulates cell–cell interaction in the NGF-induced signaling in PC12h cells. Since JSAP1 and the JNKs, especially JNK3, are highly expressed in the developing nervous system [10–12,15,21], and since JNK cascades are known to be required for many cellular processes, including apoptosis, differentiation, and cell migration [1–4,22], our finding raises the intriguing possibility that the scaffold protein JSAP1 may be involved in the coordinated regulation of cell–cell interaction and the other multiple cellular processes in the developing nervous system, by modulating JNK signaling pathways spatially and temporally.

Acknowledgments

We thank Dr. R. Fukunaga (Osaka University) for critical reading of the manuscript, and Dr. W.C. Hahn (Dana-Farber Cancer Research Institute, Harvard Medical School) for providing the retroviral vector pMKO.1-puro. This work was supported in part by grants-in-aid for scientific research from the Ministry of Culture, Sports, Science and Technology of Japan.

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