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JAB1 regulates unphosphorylated STAT3 DNA-binding activity through protein–protein interaction in human colon cancer cells

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ABSTRACT

Recent studies have revealed that unphosphorylated STAT3 forms a dimer, translocates to the nucleus, binds to the STAT3 binding site, and activates the transcription of STAT3 target genes, thereby playing an important role in oncogenesis in addition to phosphorylated STAT3. Among signaling steps of unphosphorylated STAT3, nuclear translocation and target DNA-binding are the critical steps for its activation. Therefore, elucidating the regulatory mechanism of these signaling steps of unphosphorylated STAT3 is a potential step in the discovery of a novel cancer drug. However, the mechanism of unphosphorylated STAT3 binding to the promoter of target genes remains unclear. In this study, we focused on Jun activation domain-binding protein 1 (JAB1) as a candidate protein that regulates unphosphorylated STAT3 DNA-binding activity. Initially, we observed that both unphosphorylated STAT3 and JAB1 existed in the nucleus of human colon cancer cell line COLO205 at the basal state (no cytokine stimulation). On the other hand, phosphorylated STAT3 did not exist in the nucleus of COLO205 cells at the basal state. Immunoprecipitation using nuclear extract of COLO205 cells revealed that JAB1 interacted with unphosphorylated STAT3. To investigate the effect of JAB1 on unphosphorylated STAT3 activity, RNAi studies were performed. Although JAB1 knockdown tended to increase nuclear STAT3 expression, it significantly decreased unphosphorylated STAT3 DNA-binding activity. Subsequently, JAB1 knockdown significantly decreased the expression levels of *MDR1*, *NANOG*, and *VEGF*, which are STAT3 target genes. Furthermore, the expression level of nuclear JAB1, but not nuclear STAT3, correlated with unphosphorylated STAT3 DNA-binding activity between COLO205 and LoVo cells. Taken together, these results suggest that nuclear JAB1 positively regulates unphosphorylated STAT3 DNA-binding activity through protein–protein interaction in human colon cancer cell line COLO205.

1. Introduction

Signal transducer and activator of transcription (STAT) proteins play important roles in regulating fundamental cellular processes including cell growth, differentiation, and survival. STATs are latent transcription factors that are activated by phosphorylation of a conserved tyrosine residue in response to extracellular molecules such as cytokines and growth factors. Intracellular molecules including some oncogenes and nonreceptor tyrosine kinases can also activate STATs. Following the dimerization of phosphorylated STATs, STAT dimers translocate to the nucleus where they bind to consensus binding sites within the promoter of target genes and activate the transcription of target genes [1,2]. Accumulating evidences show that, among the STATs, abnormal activation of STAT3 signaling plays a critical role in oncogenesis [3]. STAT3 is overexpressed and constitutively activated in a wide variety of human malignancies, including breast cancer, ovarian carcinoma, head and neck squamous cell carcinoma, melanoma, prostate cancer, renal cell carcinoma, pancreatic adenocarcinoma, and colon cancer [3–11]. Constitutive STAT3 dimer also induces nuclear translocation, target DNA-binding, transactivation of target genes, oncogenic transformation, and tumorigenesis [12]. Therefore, signaling steps that induce constitutive STAT3 activation can be molecular targets for the development of novel cancer drug [13].

A recent study reveals that the overexpression of unphosphorylated stat3 increased the expression levels of stat3 target genes such as *c-myc*, *c-fos*, *c-jun*, *bcl*-x, *survivin*, and*met* proto-oncogene *serine/threonine kinase 6* in stat3-null mouse embryonic fibroblasts (MEF) [14]. Furthermore, it is reported that unphosphorylated STAT3 can form the dimer [15,16], import to the nucleus by the interaction with importin-α3 [17], and bind to the STAT3 binding site [18,19]. These findings suggest that unphosphorylated STAT3 activates the transcription of STAT3 target genes and plays an

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important role in oncogenesis in addition to phosphorylated STAT3. Hence, elucidating the regulatory mechanism of unphosphorylated STAT3 activity may enhance our understanding of tumorigenesis and provide novel molecular targets for therapeutic intervention in cancers. Among the signaling steps of unphosphorylated STAT3, nuclear translocation and target DNA-binding are functionally critical steps. Therefore, disruption of the interaction between unphosphorylated STAT3 and importin-α3 may be a potential cancer treatment strategy. Target DNA-binding, a critical step for unphosphorylated STAT3 signaling, is also another target for cancer therapy; however, the regulatory mechanism of target DNA-binding remains unclear.

Jun activation domain-binding protein 1 (JAB1) was initially identified as a co-activator of activator protein 1 (AP-1) [20], and it is the fifth component of the COP9 signalosome complex [21]. An aberrant expression of JAB1 has been observed in a variety of human cancers including breast cancer, ovarian cancer, hepatocellular carcinoma, lung cancer, pancreatic cancer, and oral squamous cell carcinoma [22–28]. Numerous studies have demonstrated that JAB1 is involved in cell cycle regulation, signal transduction, DNA repair, and apoptosis [29]. JAB1 provides the binding proteins including transcription factors, with two major effects through protein–protein interaction: protein degradation and transactivation of target genes. The representative examples of the former are the degradation of $p27^{Kip1}$ and p53 proteins. $p27^{Kip1}$ and p53 proteins are exported from the nucleus to the cytoplasm and are degraded in the proteasome through the interaction with JAB1 [30,31]. The representative examples of the transactivation are the enhancement of c-Jun and hypoxia-inducible factor-1α (HIF-1α)-mediated transactivation. JAB1 stabilizes the complex of c-Jun with AP-1, and potentiates transactivation by c-Jun through protein–protein interaction [20]. JAB1 also interacts with HIF-1α and stabilize it [32,33], and enhance the transactivation by HIF-1α [32].

In this study, we have focused on JAB1 as a candidate protein that can regulate unphosphorylated STAT3 activity. To investigate the role of JAB1 in unphosphorylated STAT3 activity, we have examined the interaction between JAB1 and unphosphorylated STAT3 in the nucleus, the effect of JAB1 knockdown on unphosphorylated STAT3 DNA-binding activity and the subsequent transactivation of STAT3 target genes.

2. Materials and methods

2.1. Cell line and antibodies

Human colon cancer cell line COLO205 and LoVo were purchased from RIKEN Bioresource Center (Ibaraki, Japan). These cell lines were maintained in RPMI 1640 (COLO205) or F-12 medium (LoVo) containing glucose and supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere containing 5% CO_2. Mouse monoclonal anti-JAB1 (B-7) antibody, mouse monoclonal anti-p-STAT3 (B-7) antibody, mouse monoclonal anti-STAT3 (F-2) antibody, mouse monoclonal anti-Topo IIα (A-8) antibody, mouse monoclonal anti-PARP-1 (F-2) antibody, mouse monoclonal anti-α-Tubulin (B-5-1-2) antibody, and normal mouse immunoglobulin G (IgG, control IgG for immunoprecipitation) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Goat polyclonal anti-mouse IgG conjugated with horseradish peroxidase (HRP) was purchased from Dako (Carpinteria, CA, USA).

2.2. Preparation for nuclear extracts and cytoplasmic extracts

Nuclear and cytoplasmic extracts were prepared from COLO205 or LoVo cells using the Nuclear Complex Co-IP Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. Nuclear and cytoplasmic extracts were used for Western blotting, immunoprecipitation, and gel shift assay.

2.3. Western blotting

Cells were lysed with 1% NP-40 lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Nonidet P-40 and 1 × protease inhibitor) and incubated on ice for 30 min. After centrifugation, cell lysates containing 20 μg protein were dissolved in Laemmli buffer (25 mM Tris–HCl (pH 6.8), 0.8% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 4% (v/v) glycerol, and 0.04% (w/v) bromophenol blue) and were incubated at room temperature for 20 min. Cell lysates dissolved in Laemmli buffer were separated using SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with Tris-buffered saline containing Tween-20 (TBST) containing 0.3% milk or 1% bovine serum albumin (for detection of phosphorylated STAT3), the membrane was immunoblotted with the appropriate primary antibody, and followed by goat anti-mouse IgG conjugated with HRP. After washing, immune complexes were detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK).

2.4. Immunoprecipitation

Nuclear extracts from COLO205 cells were incubated with protein G-Sepharose beads (GE Healthcare) for 30 min at 4 °C for pre-cleaning. After centrifugation, 1 mL aliquots of the lysates containing 1 mg of protein were incubated with normal mouse IgG or anti-STAT3 antibody (1 μg) overnight at 4 °C, followed by incubation with protein G-Sepharose beads (20 μL) for 2 h at 4 °C. After washing, the beads were incubated in Laemmli buffer at 25 °C for 20 min to elute the bound proteins. After removal of the beads by centrifugation, the eluted proteins were separated by SDS-PAGE and immunoblotted with anti-JAB1 antibody or anti-STAT3 antibody.

2.5. Gel shift assay

Nuclear extracts were prepared from COLO205 cells. Gel shift assay was performed with a double-stranded, biotin-labeled oligonucleotide probe containing the consensus- binding site for STAT3 (sense strand, 5′-GATCCTTCTGGGAATTCCTAGATC-3′), using the Gelshift Chemiluminescent EMSA Kit (Active Motif) according to the manufacturer's protocol. Protein-DNA complexes were resolved on a non-denaturing polyacrylamide gel, transferred to a positively charged nylon membrane, and cross-linked to the membrane using UV cross-linker. After blocking, the membrane was incubated with the blocking buffer containing streptavidin conjugated to HRP. After washing, protein–DNA complexes were detected using a chemiluminescent substrate (Active Motif).

2.6. Reverse transcription-polymerase chain reaction

Total RNA was isolated and purified from COLO205 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Synthesis of cDNA and subsequent PCR were conducted using PrimeScript One Step RT-PCR Kit Ver. 2 (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. The sequences of primers used in this study were as follows: *MDR1*, 5′-CCTGTATTGTTTGCCACCACG-3′and 5′-ATCCACGGACACTC CTACGA-3′; *NANOG*, 5′-AACATGAGTGTGGATCCAG-3′and 5′-TCACT-CATCTTCACACGTCTTCAGGTTG-3′; *VEGF*, 5′-TCGGGCCTCCGAAACC ATGA-3′and 5′-CCTGGTGAGAGATCTGGTTC-3′;*GAPDH*, 5′-GGAAGG TGAAGGTCGGAGTC-3′and 5′-GAAGATGGTGATGGGATTTC-3′.

Reaction conditions for each primer set were 50 °C for 30 min and 94 °C for 2 min followed by 20 cycles (*GAPDH*), 26 cycles (*NANOG*), 27 cycles (*MDR1*), or 29 cycles (*VEGF*) of the following reaction: denaturing step at 94 °C for 30 s; annealing at 55 °C (*NANOG*, *GAPDH*), 58 °C (*VEGF*), or 60 °C (*MDR1*) for 30 s; and extension at 72 °C for 30 s. PCR products were resolved on a 1% agarose gel containing ethidium bromide and detected using a UV transilluminator.

2.7. Knockdown of target gene expression by siRNA transfection

For siRNA transfection, scrambled (control), or JAB1 siRNA (Thermo Scientific Dharmacon, Lafayette, CO, USA) was mixed with Lipofectamine RNAiMAX Reagent (Life Technologies, Carlsbad, CA, USA) in serum-free RPMI 1640 medium, and then the siRNA solution was incubated for 20 min at room temperature for the siRNA-cationic lipid complex to develop. Trypsinized COLO205 cells were suspended in RPMI 1640 medium containing 10% FCS and mixed with the siRNA solution (reverse transfection). Following incubation with siRNA at concentrations of 100 nM for 2 days, siRNA-transfected COLO205 cells were incubated and cultured for 3 more days. After that, the cells were harvested using a cell scraper and resuspended in PBS. Following the centrifugation at 1200 rpm for 3 min, the supernatant was removed and the siRNA-transfected cell pellet was used to prepare total cell lysate, nuclear extract, or cytoplasmic extract for Western blotting or immunoprecipitation.

2.8. Statistical analysis

Data are expressed as means ± standard deviation (S.D.). Statistical comparisons between groups were conducted using Student's *t*-test. Values of $p < 0.05$ (*) or $p < 0.01$ (**) were considered statistically significant.

3. Results

3.1. The expression levels of unphosphorylated STAT3, phosphorylated STAT3, and JAB1 in nuclear and cytoplasmic extracts from human colon cancer cell line COLO205

To investigate the expression levels of unphosphorylated STAT3, phosphorylated STAT3, and JAB1 in the nucleus and the cytoplasm of human colon cancer cells at the basal state (no cytokine stimulation), nuclear and cytoplasmic extracts from COLO205 cells were prepared, and followed by Western blotting. As shown in Fig. 1A, STAT3 and JAB1 were detected in both the nucleus and the cytoplasm. Phosphorylated STAT3 was not detected in both the nucleus and the cytoplasm. We also confirmed nuclear and cytoplasmic extracts by the detection of Topoisomerase IIα and α-Tubulin, respectively. These results suggest that unphosphorylated STAT3 and JAB1 exist in both the nucleus and the cytoplasm of COLO205 cells at the basal state.

3.2. JAB1 interacts with unphosphorylated STAT3 in the nucleus of COLO205 cells

To investigate whether JAB1 interacts with unphosphorylated STAT3 in the nucleus of COLO205 cells, nuclear extracts from COLO205 cells were immunoprecipitated with anti-STAT3 antibody, and followed by immunoblotting with anti-JAB1 antibody. As shown in Fig. 1B, endogenous JAB1 protein was detected in the proteins immunoprecipitated with anti-STAT3 antibody. By re-probing with anti-STAT3 antibody, endogenous STAT3 was found to be immunoprecipitated. These results suggest that JAB1 interacts with unphosphorylated STAT3 in the nucleus of COLO205 cells at the basal state.

3.3. JAB1 knockdown tends to increase STAT3 expression levels in the nucleus and the cytoplasm of COLO205 cells

To investigate the effect of JAB1 knockdown on STAT3 expression levels, nuclear and cytoplasmic extracts from COLO205 cells were prepared, and followed by Western blotting. JAB1 knockdown was confirmed in nuclear and cytoplasmic extracts (Fig. 2A). Moreover, JAB1 knockdown tended to increase the STAT3 expression levels in nuclear and cytoplasmic extracts (Fig. 2A). We also confirmed nuclear and cytoplasmic extracts by the detection of PARP-1 and α-Tubulin, respectively. These results suggest that JAB1 knockdown tends to increase nuclear and cytoplasmic STAT3 expression levels in COLO205 cells.

3.4. JAB1 knockdown significantly decreases unphosphorylated STAT3 DNA-binding activity and the expression levels of STAT3 target genes in COLO205 cells

JAB1 is identified as a c-Jun-interacting protein that increases c-Jun DNA-binding activity. Therefore, we hypothesized that JAB1 regulates unphosphorylated STAT3 DNA-binding activity through protein–protein interaction. To investigate this hypothesis, we performed gel shift assay using nuclear extracts prepared from COLO205 cells transfected with scrambled siRNA or JAB1 siRNA. Our results showed that JAB1 knockdown significantly decreased unphosphorylated STAT3 DNA-binding activity compared with the control (Fig. 2B and C). We further investigated the effect of JAB1 knockdown on the expression levels of STAT3 target genes such as *MDR1* [34], *NANOG* [35], and *VEGF* [36]. Total RNA from COLO205 cells transfected with scrambled siRNA or JAB1 siRNA was prepared, and followed by cDNA synthesis and RT-PCR. Our results showed that JAB1 knockdown significantly decreased the expression levels of *MDR1*, *NANOG*, and *VEGF* of STAT3 targeted genes (Fig. 3A–D). Taken together, these results suggest that JAB1 regulates unphosphorylated STAT3 DNA-binding activity and the expression levels of STAT3 target genes in COLO205 cells.

3.5. Nuclear JAB1 expression level correlates with unphosphorylated STAT3 DNA-binding activity between COLO205 and LoVo cells

Furthermore, to investigate whether JAB1 expression level correlates with unphosphorylated STAT3 DNA-binding activity, nuclear and cytoplasmic extracts were prepared from COLO205 and LoVo cells, and followed by Western blotting and gel shift assay. The comparison between COLO205 and LoVo cells revealed that the expression level of nuclear JAB1, but not cytoplasmic JAB1, correlated with STAT3 DNA-binding activity (Fig. 4A, B, C, E, and F). Nuclear STAT3 expression level did not correlate with STAT3 DNA-binding activity (Fig. 4A, D, E, and F). In addition, phosphorylated STAT3 was not detected in both COLO205 and LoVo cells (data not shown). These results indicate that nuclear JAB1 expression level correlates with unphosphorylated STAT3 DNA-binding activity.

4. Discussion

Recent studies demonstrate that not only phosphorylated STAT3 but also unphosphorylated STAT3 can form the dimer, translocate to the nucleus, and bind to the STAT3 binding sites, thereby activating the transcription of target genes [14–19]. Consistent with these findings, we observed that unphosphorylated STAT3 existed in the nucleus, bound to target DNA, and activated the

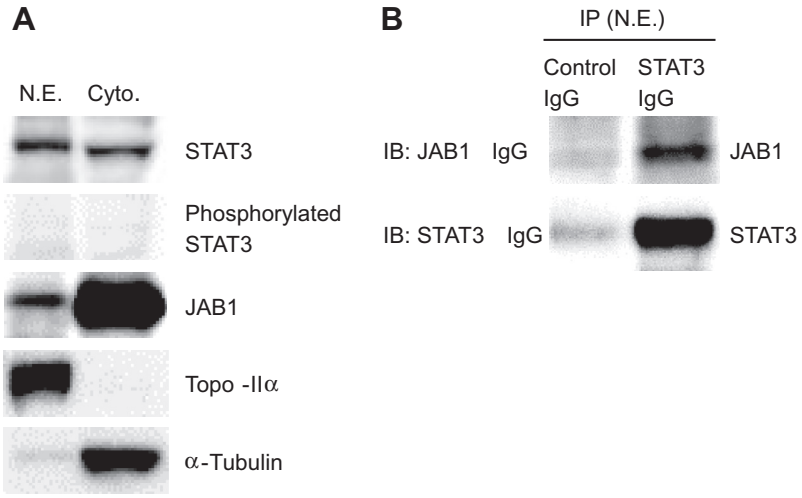

Fig. 1. The expression levels of STAT3, phosphorylated STAT3, JAB1, and the interaction between JAB1 and STAT3 in the nucleus. (A) Nuclear and cytoplasmic extracts were prepared using the human colon cancer cell line COLO205, and followed by Western blotting with anti-STAT3, anti-phosphorylated STAT3, anti-JAB1, anti-Topoisomerase IIα or anti-α-Tubulin antibody. N.E., nuclear extracts; Cyto., cytoplasmic extracts. (B) Nuclear extracts were prepared from COLO205 cells, and the extracts were immunoprecipitated with normal mouse antibody or anti-STAT3 antibody, followed by Western blotting with anti-JAB1 or anti-STAT3 antibody. IP (N.E.), immunoprecipitation using nuclear extracts. IB, immunoblotting. Control IgG, normal mouse IgG.

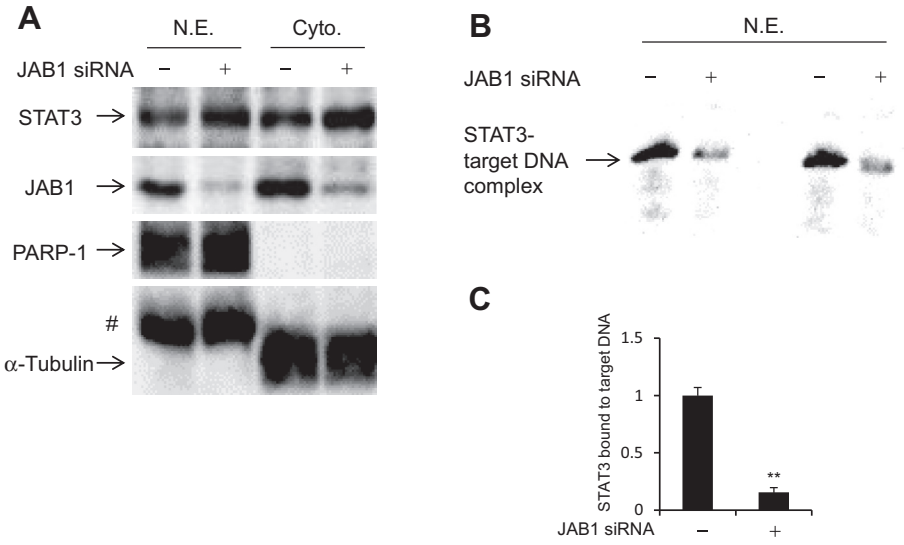

Fig. 2. Effect of JAB1 knockdown on STAT3 expression levels and its DNA-binding activity. (A) Nuclear and cytoplasmic extracts were prepared from COLO205 cells transfected with scrambled siRNA or JAB1 siRNA. STAT3, JAB1, PARP-1, and α-Tubulin in nuclear and cytoplasmic extracts were detected by Western blotting with anti-STAT3, anti-JAB1, anti-PARP-1, and anti-α-Tubulin antibody, respectively. Hash mark (#) in the lowest panel indicates cleaved PARP-1 in nuclear extracts. (B) Nuclear extracts were prepared from COLO205 cells transfected with scrambled siRNA or JAB1 siRNA. STAT3 bound to biotin-labeled target DNA was detected by a gel shift assay using nuclear extracts. (C) The intensity of each band was quantified using ImageJ software. The ratio of STAT3 bound to biotin-labeled DNA probe to the amount of nuclear extracts used in each reaction was normalized to the values in control cells. Each bar represents the mean ± S.D. of three independent experiments. $^{**}p$ <0.01, significantly different (n = 3).

transcription of STAT3 target genes in human colon cancer cell line COLO205 at the basal state. A previous study reveals that STAT3 is translocated into the nucleus by the interaction with importin-α3 in a STAT3 phosphorylation-independent manner [17]. It is possible that nuclear import of unphosphorylated STAT3 is mediated by the interaction with importin-α3 in COLO205 cells. In addition to nuclear import, target DNA-binding is a critical step for the activation of transcription factors. In this study, we have identified JAB1 as a novel regulator for unphosphorylated STAT3 DNA-binding activity through protein–protein interaction. JAB1 knockdown decreased unphosphorylated STAT3 DNA-binding activity, but tends to increase nuclear STAT3 expression level. Furthermore, we observed that the expression level of nuclear JAB1, but not nuclear STAT3, correlates with unphosphorylated STAT3 DNA-binding activity between COLO205 and LoVo cells. Taken together, these results suggest that nuclear JAB1 regulates unphosphorylated STAT3 DNA-binding activity through protein–protein interaction in a nuclear STAT3 expression level-independent manner. Interestingly, it has been reported that JAB1 knockdown stabilized MYC protein level, but inhibited MYC-induced transcriptional activity [37], suggesting that JAB1 increased the turnover of MYC protein and promoted the transcription of MYC target genes. Based on the above observations, there is a possibility that JAB1 may regulate nuclear STAT3 expression level by promoting protein degradation of the STAT3, which is not bound to the target DNA. Further investigations are needed to elucidate how JAB1 determines the fate of the transcription factors as its binding partner: stable binding to target DNA or protein degradation. Moreover, recent study

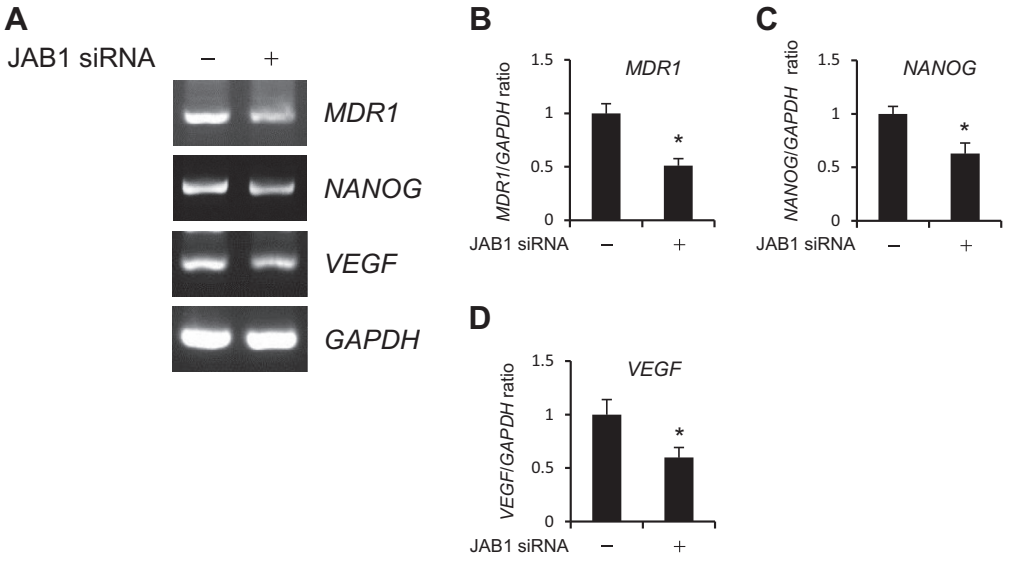

Fig. 3. Effect of JAB1 knockdown on the expression levels of STAT3 target genes. (A) Total RNA was purified from COLO205 cells transfected with scrambled siRNA or JAB1 siRNA, and followed by cDNA synthesis. The expressions of *MDR1*,*NANOG*,*VEGF*, and *GAPDH* were detected using RT-PCR. (B–D) The intensity of each band was quantified using ImageJ software. The ratio of *MDR1*, *NANOG*, or *VEGF* to *GAPDH* was normalized to the values in control cells. Each bar represents the mean ± S.D. of three independent experiments. $^{*}p$ <0.05, significantly different (n = 3).

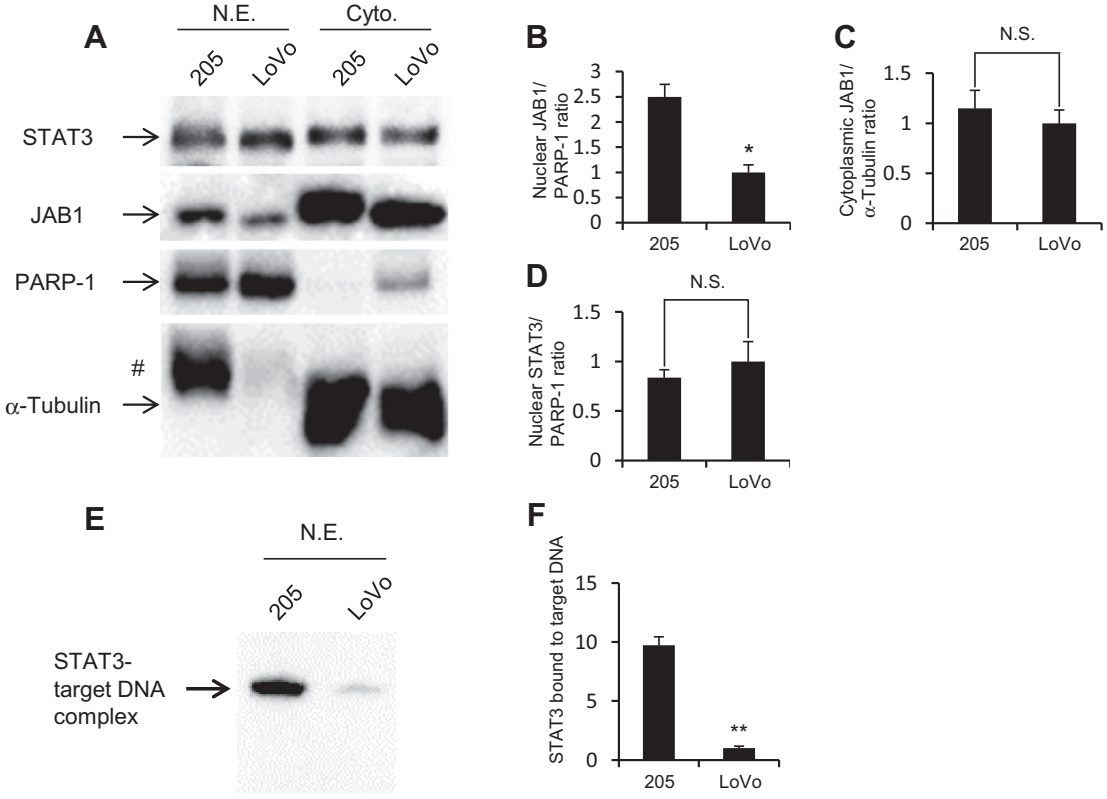

Fig. 4. Comparison of nuclear and cytoplasmic expression levels of STAT3, JAB1, and STAT3 DNA-binding activity between COLO205 and LoVo cells. (A) Nuclear and cytoplasmic extracts were prepared from COLO205 and LoVo cells. STAT3, JAB1, PARP-1, and α-Tubulin in nuclear and cytoplasmic extracts were detected by Western blotting with anti-STAT3, anti-JAB1, anti-PARP-1, and anti-α-Tubulin antibody, respectively. Hash mark (#) in the lowest panel indicates cleaved PARP-1 in nuclear extracts. (B, C, and D) The intensity of each band was quantified using ImageJ software. The ratio of nuclear JAB1 or nuclear STAT3 to PARP-1 was normalized to the values in LoVo cells (B and D). The ratio of cytoplasmic JAB1 to α-Tubulin was normalized to the values in LoVo cells (C). Each bar represents the mean ± S.D. of three independent experiments. N.S., no significant difference (n = 3). $^{*}p$ <0.05, significantly different (n = 3). (E) Nuclear extracts were prepared from COLO205 and LoVo cells. STAT3 bound to biotin-labeled target DNA was detected by a gel shift assay using nuclear extracts. (F) The intensity of each band was quantified using ImageJ software. The ratio of STAT3 bound to biotin-labeled DNA probe to the amount of nuclear extracts used in each reaction was normalized to the values in LoVo cells. Each bar represents the mean ± S.D. of three independent experiments. $^{**}p$ <0.01, significantly different (n = 3).

reveals that STAT3 regulates JAB1 expression in mammary carcinoma cells [38]. STAT3 knockdown reduces JAB1 promoter activity, JAB1 mRNA, and JAB1 protein expression levels. STAT3 overexpression reversely increases JAB1 expression [38]. These findings and our data suggest that JAB1 closely relates to STAT3, and JAB1-STAT3 axis may play an important role in oncogenesis.

In this study, we identified JAB1 as a positive regulator for unphosphorylated STAT3 DNA-binding activity through protein–protein interaction in human colon cancer cell line COLO205. Furthermore, the detailed analyses of the relationship between JAB1 and multiple transcription factors containing STAT3 related to tumorigenesis may enhance our understanding of their regulatory mechanism by JAB1 and lead to the development of an effective cancer therapeutic drug.

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