Requirement of JNK Signaling for Self-renewal and Tumor-initiating Capacity of Ovarian Cancer Stem Cells

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Abstract. *Background/Aim: Activation of the c-JUN N-terminal kinase (JNK) signaling pathway has been associated with poor survival of ovarian cancer patients, but the role(s) and significance of JNK signaling in ovarian cancer cells remain poorly understood. In the present study, we aimed to investigate the role of JNK specifically in ovarian cancer stem cells (CSCs). Materials and Methods: The effect of JNK inhibition on the self-renewal (CSC marker expression, sphere-forming ability) and tumor-initiating capacity was examined in CSCs derived from the A2780 human ovarian cancer cell line. JNK inhibition was achieved either pharmacologically or genetically by use of RNA interference. Results: Both pharmacological and genetic targeting of JNK resulted in loss of self-renewal and tumor-initiating capacity of A2780 CSCs. Conclusion: Our findings demonstrate, to our knowledge for the first time, that JNK has a pivotal role in the maintenance of ovarian CSCs.*

Ovarian cancer, one of the most common and lethal gynecological malignancies, is the fifth leading cause of cancer death in women worldwide (1). Typically, patients with ovarian cancer present with advanced-stage disease, for which maximal cytoreductive, debulking surgery followed by chemotherapy is the mainstay of therapy (2). Ovarian carcinomas are highly chemosensitive in general, yet recurrence is inevitable in the majority of cases, even after apparently successful initial treatment and is usually fatal (3). Extensive efforts have been and are being made, to develop novel strategies to treat recurrent disease (4). However, the prognosis of recurrent ovarian cancer still remains quite poor (3), which might imply that attention should be directed not only to treatment but also to prevention of recurrence.

Intriguingly, it does not rarely happen that recurrent ovarian carcinomas are sensitive, at least initially, to the same chemotherapeutic agents used for the primary treatment (3), suggesting that the primary and recurrent tumors are similarly heterogeneous in terms of chemoresistance, *i.e.* consisting of both chemosensitive and chemoresistant tumor cell populations. Such a clinical picture of ovarian cancer is more consistent with and better explained by the cancer stem cell (CSC) model, which assumes the existence of a small sub-population of therapy-resistant tumor cells endowed with the capacity to recapitulate the heterogeneity of the original tumor (5, 6), than by simple amplification of chemoresistant clones surviving chemotherapy. Accordingly, targeting and elimination of the CSC population of ovarian cancer is expected to contribute to improved survival of patients through prevention of fatal recurrence. However, although the molecular biology of ovarian CSCs is only now beginning to be understood (7-9), much still remains to be elucidated to identify druggable molecular targets in order to effectively control ovarian CSCs in the clinical setting.

Notably, de-regulation of the c-JUN N-terminal kinase (JNK) signaling pathway has been documented in a variety of human cancer types, including ovarian cancer, and recent studies have revealed that JNK pathway activation is

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associated with shorter progression-free survival of patients with ovarian cancer (10-12). These findings suggested that JNK signaling might have a significant role in the biology of ovarian cancer, yet its exact role(s) in ovarian cancer cell biology still remains unclear. In the present study, we investigated the role of JNK signaling in CSCs derived from A2780 ovarian cancer cells. Our data show that JNK has an essential role in the maintenance of the self-renewal and tumor-initiating capacity of ovarian CSCs.

Materials and Methods

Antibodies and reagents. Anti-c-JUN (#9165), anti-phospho-c-JUN (#9261), anti-phospho-JNK (#9251), anti-SOX2 (#3579), anti-NANOG (#4903) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-β-actin (A1978) was from Sigma (St. Luis, MO, USA). Anti-JNK1 (sc-474) and anti-JNK2 (sc-7345) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-NESTIN (MAB5326) was purchased from Millipore (Billerica, MA, USA). Anti-CD133 (W6B3C1) was from Miltenyi Biotech (Germany). A chemical inhibitor of JNK, SP600125, was purchased from Calbiochem (La Jolla, CA, USA) and was dissolved in dimethyl sulfoxide (DMSO) to prepare a 50 mM stock solution.

Cell culture. The parental human ovarian cancer cell line A2780, a kind gift from Dr. T Tsuruo at the Institute of Molecular and Cellular Biosciences, the University of Tokyo, and Drs. RF Ozols and TC Hamilton at the National Institutes of Health (13, 14), was maintained in Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg/ml streptomycin. To establish A2780 CSCs, the parental A2780 cells were cultured in the stem cell culture medium on non-coated dishes to promote sphere formation (15, 16). Cells from spheres formed under this culture condition were then amplified under the monolayer stem cell culture condition (15, 16) and implanted subcutaneously into female nude mice (Clea Japan, Inc., Tokyo, Japan). After formation of subcutaneous tumors, the primary tumors were subjected to serial transplantation for secondary tumor formation, and tumor cells from a secondary tumor were cultured in stem cell culture medium on non-coated dishes. Cells from spheres formed under this culture condition were amplified under the monolayer the stem cell culture condition, which were then used as A2780 CSCs after characterization as shown in Figure 1. The authenticity of A2780 CSCs as cells derived from A2780 was verified by genotyping of short tandem repeat (STR) loci (Bio-Synthesis, Inc., Lewisville, TX, USA) followed by comparison to the ATCC STR database (http://www.atcc.org/STR_Database.aspx) for Human Cell Lines. Unless otherwise indicated, A2780 CSCs were stably maintained and used for experiments under the monolayer stem cell culture condition. In principle, the stem cell culture medium [DMEM/F12 medium supplemented with 1% B27 (Gibco-BRL, Carlsbad, CA, USA), 20 ng/ml epidermal growth factor (EGF) and fibroblast growth factor (FGF2) (Peprotech, Inc., Rocky Hill, NJ, USA, D-(+)-glucose (final concentration, 26.2 mM), L-glutamine (final concentration, 4.5 mM), 100 units/ml penicillin and 100 μg/ml streptomycin] was changed every three days, and EGF and FGF2 were added to the culture medium every day. Throughout the study, the cell number was determined using a hemocytometer, and cellular viability was examined by the dye exclusion method (0.2% trypan blue). Cellular viability (%) was defined as 100× number of viable cells/(number of viable cells + number of dead cells).

Gene silencing by siRNA. siRNAs against human *JNK1* (#1: VHS40722, #2: VHS40724) and *JNK2* (#1: VHS40726, #2: VHS40729) as well as Medium GC Duplex #2 of Stealth RNAi™ siRNA Negative Control Duplexes (as a control for siRNA experiments) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Transfection of siRNAs was performed using Lipofectamine RNAiMAX™ (Life Technologies) according to the manufacturer's instructions. To achieve sustained knockdown of the target genes, siRNA transfection was repeated four days after the initial transfection.

Sphere-formation assay. After being dissociated into single cells, A2780 CSCs were serially diluted in the stem cell culture medium and seeded into non-coated 96-well plates so that there was a single cell in each well. Wells containing a single cell were marked on the next day and, six days after seeding, the percentage of marked wells with a sphere relative to the total number of marked wells was determined.

Flow cytometric analysis. Dissociated cells were washed with ice-cold phosphate-buffered saline (PBS), fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature, and washed again with PBS twice, followed by incubation with the antibody to CD133 in FACS buffer (0.5% w/v bovine serum albumin, 0.1% w/v NaN_3 in PBS) for 1 h and then with Alexa Fluor® 488 goat anti-mouse IgG (Life Technologies) for another 30 min at room temperature. Gating for single cells was established using forward scatter in the isotype control samples. The isotype control samples were used to establish a gate in the fluorescein isothiocyanate channel. Cells showing signal for CD133 above the gate established by the isotype control were deemed CD133-positive. All flow cytometric experiments were run on FACSCanto™ II Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the data were analyzed using FlowJo software, version 7.6.5 (Treestar Inc., Ashland, OR, USA).

Immunoblot analysis. Cells were washed with ice-cold PBS and lysed in RIPA buffer [10 mM Tris-HCl (pH 7.4), 0.1% SDS, 0.1% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1.5 mM Na_3VO_4, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate and 1% protease inhibitor cocktail set III (Calbiochem)]. After centrifugation for 10 min at 14,000× *g* at 4˚C, the supernatants were recovered as the cell lysates, and the protein concentration of the cell lysates was determined by BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Cell lysates containing equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane was probed with a primary antibody against Sex determining region Y-box 2 [SOX2], Tir Na Nog [NANOG], NESTIN, phospho-c-JUN, c-JUN, JNK1, JNK2 or β-Actin, and then with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody according to the protocol recommended by the manufacturer of each antibody. Immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).

Immunofluorescence analysis. A2780 CSCs were washed with ice-cold PBS and spun-down onto glass slides by using StatSpin®

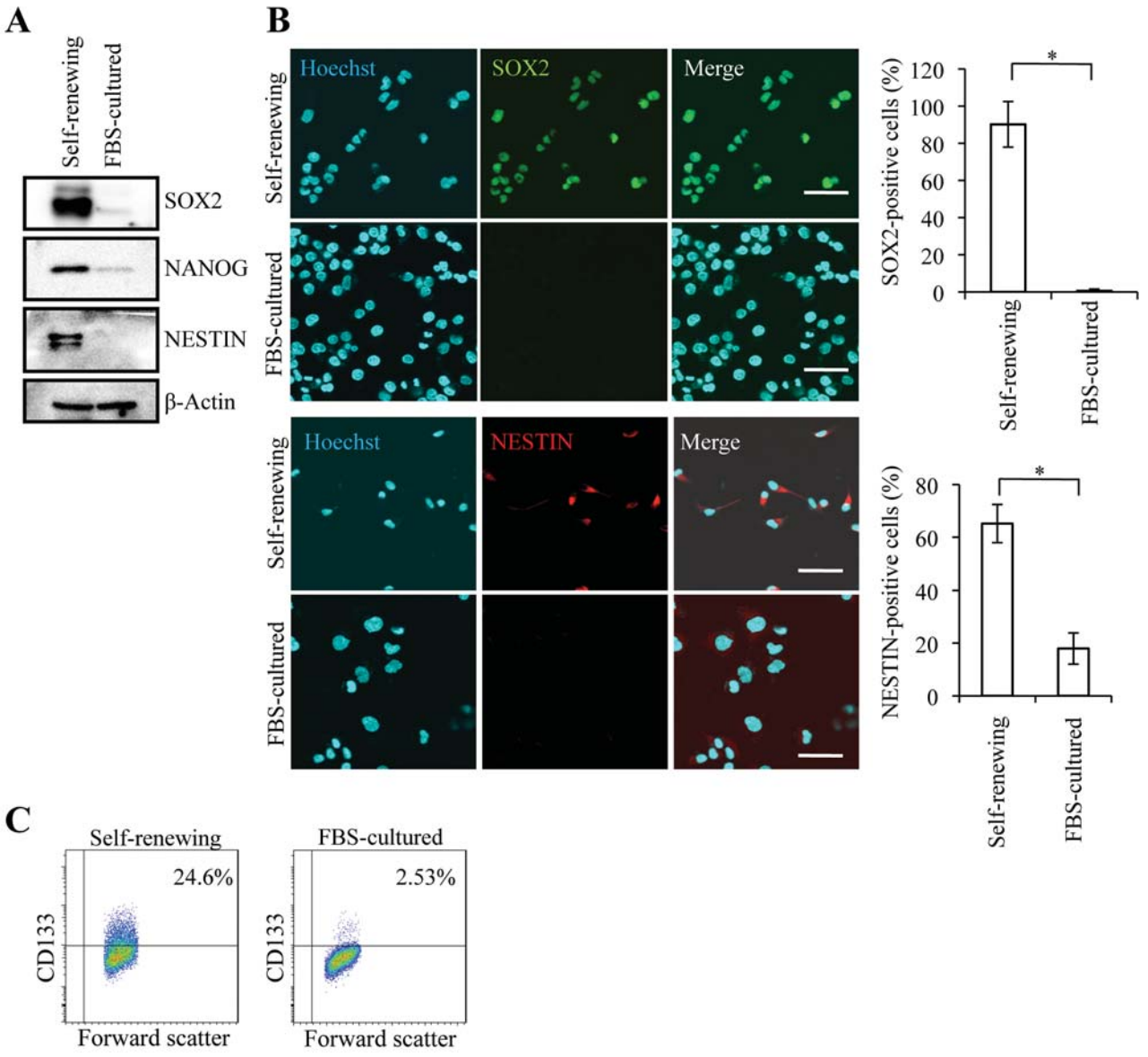

Figure 1. *Characterization of A2780 cancer stem cell (CSC). A2780 CSCs cultured under stem cell culture conditions (Self-renewing) and serum culture conditions (fetal bovine serum [FBS]-cultured) were subjected to immunoblot analysis of SOX2, NANOG, NESTIN and β-Actin (A) and immunofluorescence analysis of SOX2 and NANOG (B). The values in the graphs represent means±SD of the independent experiments. *p<0.05. Scale bars, 50 μm. C: Self-renewing and FBS-cultured A2780 CSCs were subjected to flow cytometric analysis of the cell surface expression of CD133. Representative flow cytometric plots are shown together with the percentages of CD133-positive cells.*

Cytofuge (Iris Sample Processing, Inc. Westwood, Billerica, MA, USA) for 5 min at 3,200×rpm at room temperature. FBS-cultured cells were seeded directly onto coverslips. After fixation with 4% formaldehyde in PBS for 10 min, the cells were permeabilized and blocked with 0.4% Triton X-100/2% FBS in PBS for 15 min at room temperature. After being washed with PBS three times, the cells were incubated with a primary antibody in PBS containing 2% FBS at 4˚C overnight and then with Alexa Fluor 488-conjugated secondary antibody in the same buffer for 1 h at room temperature, followed by counterstaining with Hoechst 33342 (Life Technologies) to label nuclei. Fluorescence images were acquired using a confocal laser-scanning microscope (FLUOVIEW FV10i: Olympus, Tokyo, Japan).

Mouse studies. Subcutaneous implantation of tumor cells was carried out essentially as previously described elsewhere (17). For intraperitoneal implantation, A2780 CSCs (2×10^6 viable cells) were suspended in 200 μl of PBS after determination of cellular viability

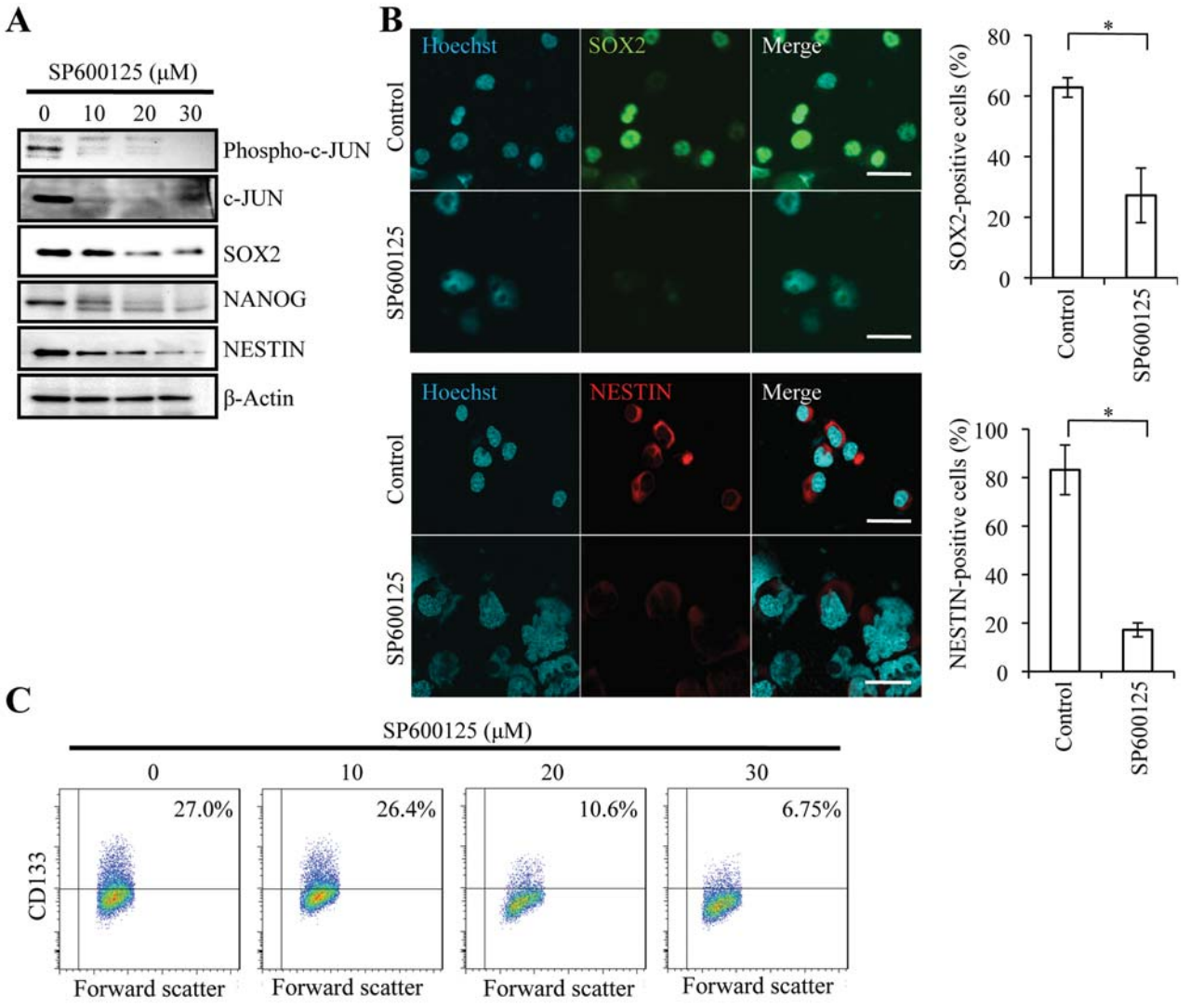

Figure 2. *Pharmacological inhibition of c-JUN N-terminal kinase (JNK) by SP600125 causes loss of the stem cell marker expression in A2780 cancer stem cells (CSCs). A: A2780 CSCs treated with or without the indicated concentration of SP600125 for three days were subjected to immunoblot analysis of phospho-c-JUN, c-JUN, SOX2, NANOG and β-Actin. B: A2780 CSCs treated with or without SP600125 (20 μM) for three days were subjected to immunofluorescence analysis of the same proteins. The values in the graphs represent means±SD of three independent experiments. *p<0.05. Scale bars, 50 μm. C: A2780 CSCs treated as in (A) were subjected to flow cytometric analysis of the cell surface expression of CD133. Representative flow cytometric plots with the percentages of CD133-positive cells are shown.*

and injected into the peritoneal cavity of 5- to 8-week-old female BALB/cAJcl-nu/nu mice (Clea Japan, Inc., Tokyo, Japan). The animal experiments conducted in this study were performed under the protocol approved by the Animal Research Committee of Yamagata University (No. 25075).

Statistical analysis. Results are expressed as the means and standard deviation (SD), and differences were compared using the 2-tailed Student's *t*-test. Mouse survival was evaluated by the Kaplan–Meier method and analyzed by using the log-rank test. *p*-Values of less than 0.05 were considered statistically significant and indicated with asterisks in the figures.

Results

Establishment of CSCs from the A2780 human ovarian cancer cell line. To examine the possible role of JNK in ovarian CSCs, we first sought to isolate CSCs from ovarian cancer xenografts formed by implantation of human ovarian cancer cell lines. Since sphere-forming ability has been closely associated with ovarian CSCs (18, 19), we attempted to selectively isolate sphere-forming cells from the xenografts and thereby established CSCs of the A2780 cell

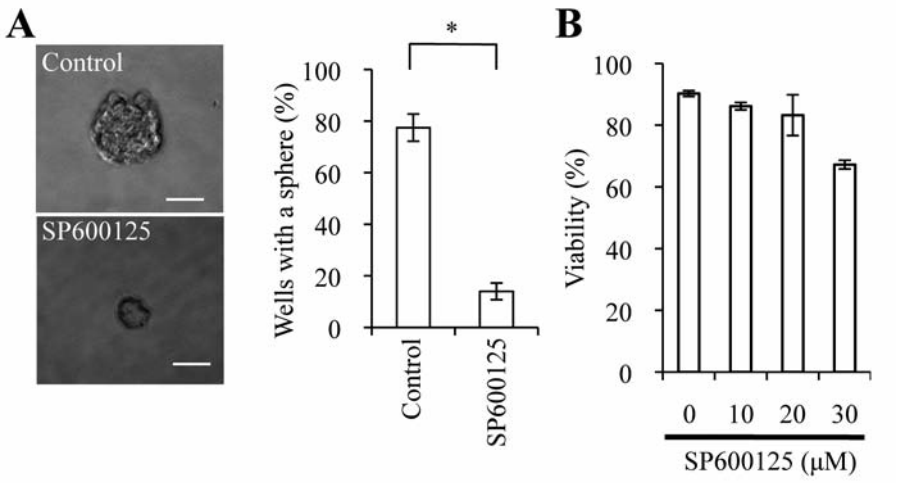

Figure 3. *The effect of SP600125 treatment on the sphere-forming ability and viability of A2780 cancer stem cells (CSCs). A: A2780 CSCs treated with or without SP600125 (20 μM) for three days were subjected to the sphere-formation assay in the absence of SP600125 after washout of SP600125. Right, The graph shows the percentage of wells in which a tumor sphere was formed from a single cell; the values represent means±SD from three independent experiments. *p<0.05. Left, Photographs of representative wells. Scale bars, 50 μm. B: A2780 CSCs treated with or without the indicated concentration of SP600125 for three days were subjected to determination of the cell viability by dye exclusion. The values in the graph represent means±SD of three independent experiments.*

line (A2780 CSCs) successfully, as detailed in the Materials and Methods Section. Compared to the parental A2780 cells, the A2780 CSCs established in this study expressed higher levels of stem cell markers for ovarian cancer such as SOX2, NANOG, and NESTIN (20, 21), which was confirmed both by immunoblot and immunocytochemical analyses (Figure 1 A and B). Consistent with this, flow cytometric analyses confirmed that the A2780 CSCs were also enriched for cells with surface expression of CD133, also known to be an ovarian CSC marker (7-9) (Figure 1 C). Thus, the data indicate that the A2780 CSCs established herein are enriched in self-renewing cells with stem cell-like properties.

Pharmacological inhibition of JNK results in loss of the self-renewal capacity of A2780 CSCs. To determine the role of JNK signaling in ovarian CSCs, we tested the effect of a JNK inhibitor SP600125 on the self-renewal capacity and viability of A2780 CSCs. SP600125 inhibited the activity of JNK in A2780 CSCs in a concentration-dependent manner, as assessed by the expression of phosphorylated c-JUN. In parallel with the reduced JNK activity, the expression of SOX2, NANOG, and NESTIN were decreased with increasing concentration of SP600125 (Figure 2A). Consistent with the immunoblot data, immunocytochemical analysis showed that the proportions of cells positive for SOX2 and NESTIN, which are stem cell markers expressed in the nucleus and cytoplasm, respectively, were reduced by SP600125 treatment at 20 μM (Figure 2B). We further confirmed that the proportion of CD133-positive cells was also decreased by SP600125 treatment of A2780 CSCs in a concentration-dependent manner (Figure 2C). The results of the sphere formation (Figure 3A) and cell viability (Figure 3 B) assays demonstrated that 3-day pretreatment of A2780 CSCs with 20 μM SP600125 did not affect their viability appreciably but significantly inhibited their ability to form spheres in the absence of SP600125. Thus, the data suggested that SP600125 at this concentration specifically inhibited the self-renewal capacity, but not the viability itself of A2780 CSCs.

Knockdown of JNK results in loss of the self-renewal capacity of A2780 CSCs. The results of the pharmacological inhibitor experiments suggested that JNK activity is required for the maintenance of the self-renewal capacity of A2780 CSCs. To definitively determine whether JNK plays an essential role in their maintenance, we next tested the effect of JNK knockdown on A2780 CSCs. A2780 CSCs were transiently transfected with siRNAs against *JNK1* and *JNK2* simultaneously to effectively inhibit the JNK activity. Two different combinations of siRNAs were used in this study, both of which reduced, albeit partially, the expression of JNK1, JNK2 and phosphorylated c-JUN (Figure 4A). Under the knockdown condition, the two combinations of siRNAs both inhibited the self-renewal capacity of A2780 CSCs, as represented by stem cell marker expression (Figure 4A and B) and sphere-forming ability (Figure 4C), in total agreement with the results of the inhibitor experiments. Combined, the results of the pharmacological and genetic JNK inhibition

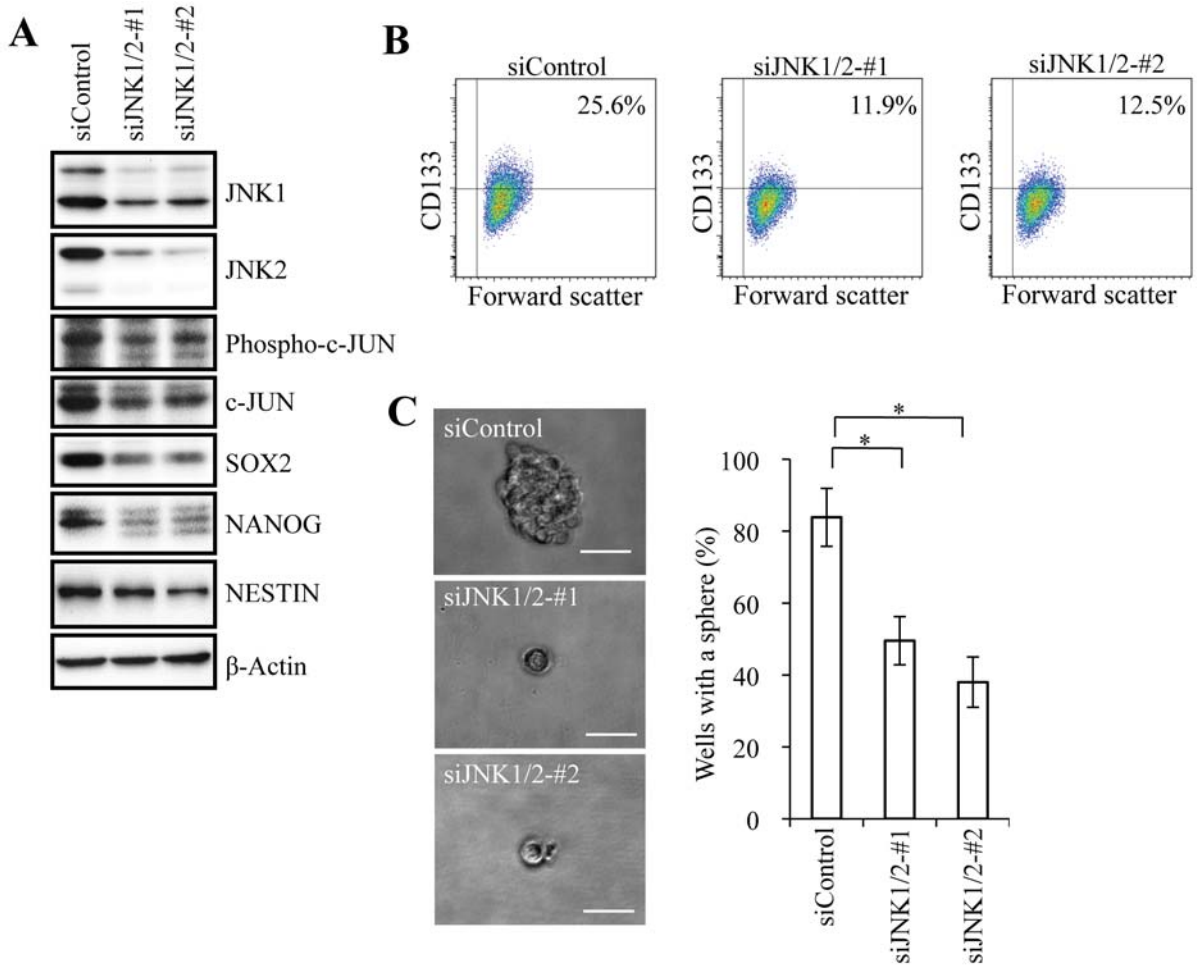

Figure 4. *c-JUN N-terminal kinase (JNK) knockdown causes loss of the self-renewal capacity of A2780 cancer stem cells (CSCs). A: A2780 CSCs were transiently transfected with two combinations (#1 and #2) of siRNAs against JNK1 and JNK2 (siJNK1/2), or with a control siRNA (siControl), as detailed in the Materials and Methods. After eight days, the transfected cells were subjected to immunoblot analysis of JNK1, JNK2, phospho-c-JUN, c-JUN, SOX2, NANOG, NESTIN and β-Actin. B: A2780 CSCs treated as in (A) were subjected to flow cytometric analysis for the cell surface expression of CD133. Representative flow cytometric plots are shown together with the percentages of CD133-positive cells. C: A2780 CSCs treated as in (A) were subjected to the sphere-formation assay. Right, The graph shows the percentage of wells in which a tumor sphere was formed from a single cell; the values represent means±SD from three independent experiments. *p<0.05. Left, Photographs of representative wells. Scale bars, 50 μm.*

analyses indicate that JNK is required for A2780 CSCs to self-renew as cells with stem cell-like properties.

JNK activity is essential for the maintenance of the tumor-initiating capacity of A2780 CSCs. Tumor-initiating capacity is one of the critical features that characterize CSCs. We therefore next examined if JNK also plays a key role in the maintenance of the tumor-initiating capacity of A2780 CSCs. We confirmed in our pilot experiment that intraperitoneal implantation of 2×10^6 A2780 CSCs into nude mice results in formation of disseminated tumors within the peritoneal cavity and subsequently in death of the recipient mice ~50 days after implantation. We, therefore, used this intraperitoneal xenograft model of ovarian cancer and tested the effect of JNK inhibition on the tumor-initiating capacity of A2780 CSCs. A2780 CSCs were treated either with 20 μM SP600125 or control vehicle for three days, after which 2×10^6 of the treated cells were implanted intraperitoneally into nude mice. Strikingly, whereas all five mice implanted with vehicle-treated A2780 CSCs developed intraperitoneal tumors and died within two months, four out of the five mice implanted with SP600125-treated cells were alive without any signs of tumor development even at five months after implantation (Figure 5). Thus, the results suggest that JNK inhibition causes substantial loss of the tumor-initiating capacity of A2780 CSCs.

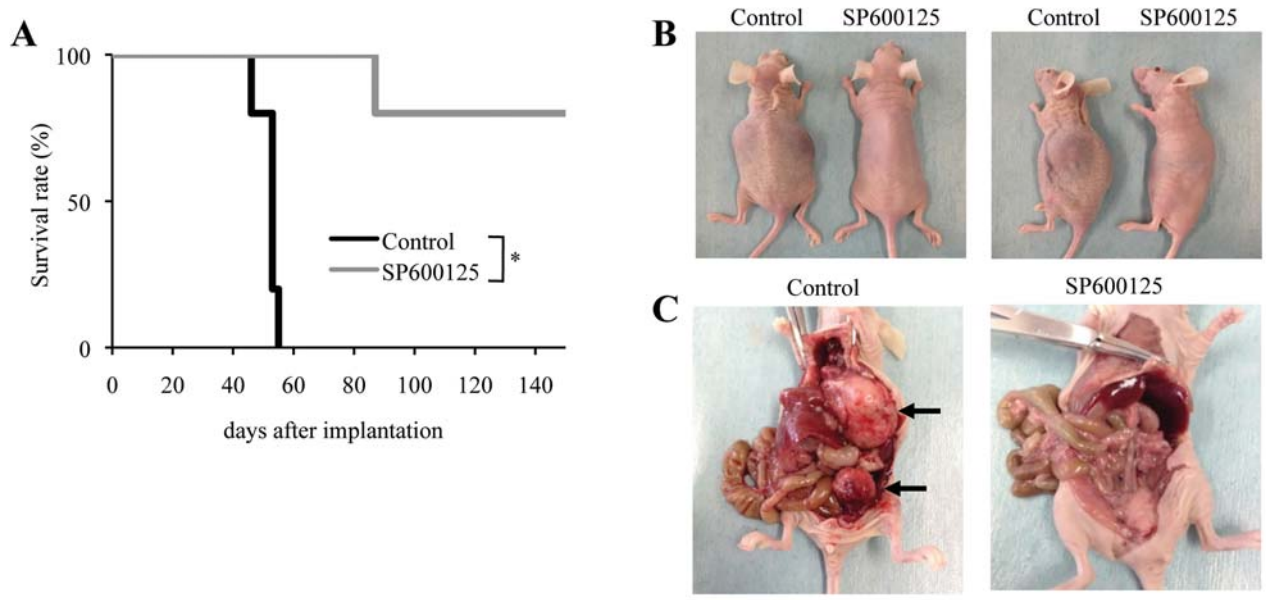

Figure 5. *c-JUN N-terminal kinase (JNK) activity is required for the maintenance of the tumor-initiating capacity of A2780 cancer stem cells (CSCs). A: Mice were implanted intraperitoneally with 2×10^6 viable A2780 CSCs treated without or with SP600125 (20 μM) for three days. Kaplan–Meier survival curves of the mice (n=5 for each group) are shown. *p<0.01. Mice treated as in (A) (but separately from those for survival analysis) were sacrificed for autopsy on day 54 to determine the extent of intraperitoneal tumor burden, and photographed just prior to (B; Left: top view, Right: side view) and at (C) autopsy. Intraperitoneal tumors are indicated with arrows.*

Discussion

Previous studies have demonstrated that JNK pathway activation occurs frequently in ovarian cancer and is also associated with shorter progression-free survival (10, 12), giving rise to the idea that activated JNK signaling may have a role in progression of ovarian cancer. Indeed, one of these studies also suggested that JNK may be involved in the control of proliferation and survival of serum-cultured, hence most likely non-stem, ovarian cancer cells (12). However, to date, the role of JNK in ovarian CSCs has remained uninvestigated. Herein, we have for the first time shed light on the potential role of JNK in ovarian CSCs. We have demonstrated that inhibition of JNK activity in A2780 CSCs causes loss of their capacity to self-renew as cells with stem cell marker expression and sphere-forming ability, as well as loss of their capacity to initiate tumor formation. Thus, our data suggest that intact JNK signaling is essential for the maintenance of ovarian CSCs. Given the presumed role of CSCs as a main culprit in tumor recurrence (22-24), our findings may lend support to the intriguing possibility that activated JNK signaling contributes to ovarian cancer progression through maintenance of therapy-resistant CSCs and that therapeutic targeting of JNK may become a promising approach to improve the prognosis of ovarian cancer through control of CSCs.

Quite importantly from a therapeutic perspective, we have previously demonstrated that JNK is required *in vivo* for the maintenance of CSCs residing in pre-established glioblastoma tumors and that systemic JNK inhibitor administration quite effectively depletes the CSC population within the tumors (16). Of equal importance, at the same time we demonstrated that the JNK inhibitor treatment protocol had no discernible adverse effects on the general health status and cognitive function of the recipient mice during the long-term follow-up observation (up to approximately two years) (16). These findings clearly indicated that JNK could be a viable molecular target for CSC-directed therapy against cancer whose CSC maintenance is dependent on JNK signaling. Meanwhile, the present study showed that the ovarian CSCs derived from A2780 were comparably sensitive to JNK inhibition *in vitro* when compared to the glioblastoma CSCs analyzed *in vitro* in previous studies (16, 25). Together, these current and previous findings suggest that JNK could be a viable therapeutic target for controlling CSCs of not only glioblastoma but also ovarian cancer, and therefore further study is warranted to investigate the potential of JNK inhibitors to eliminate ovarian CSCs *in vivo* and to prevent tumor recurrence in pre-clinical animal models of ovarian cancer.

Besides ovarian cancer, aberrant activation of the JNK signaling pathway has been observed in a variety of human cancer types, including glioblastoma and non-small cell lung

cancer (11, 26). Despite such prevalence in human cancer, the pathophysiological significance of JNK activation remains unclear. Herein, we propose a novel role of JNK in the maintenance of ovarian CSCs. Although we recently reported that JNK is required for the maintenance of tumor-initiating capacity of non-small cell lung cancer cells, we were unable to test the role of JNK in the maintenance of the self-renewal capacity of CSCs of NSCLC in that study (17). On this regard, the current study provides, as for as we are aware far the first time, unequivocal evidence that the mechanism of JNK-dependent maintenance of the CSC properties is also operative in non-glial human cancer, demonstrating that the mechanism is shared by other types of human cancer instead of being unique to glioblastoma. Future studies will clarify how prevalent the role of JNK in CSC maintenance is in human cancer in general, and accordingly, the significance of JNK as a therapeutic target in CSC-directed cancer therapy.

Conflicts of Interest

The Authors declare no conflicts of interest.

Acknowledgements

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