GSKJ4, A Selective Jumonji H3K27 Demethylase Inhibitor, Effectively Targets Ovarian Cancer Stem Cells

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Abstract. *Background/Aim: Global increase in the trimethylation of histone H3 at lysine 27 (H3K27me3) has been associated with the differentiation of normal stem cells and cancer cells, however, the role of H3K27me3 in the control of cancer stem cells (CSCs) remains poorly understood. We investigated the impact of increased H3K27me3 on CSCs using a selective H3K27 demethylase inhibitor GSKJ4. Materials and Methods: The effect of GSKJ4 on the viability as well as on the self-renewal and tumor-initiating capacity of CSCs derived from the A2780 human ovarian cancer cell line was examined. Results: GSKJ4 induced cell death in A2780 CSCs at a concentration non-toxic to normal human fibroblasts. GSKJ4 also caused loss of self-renewal and tumor-initiating capacity of A2780 CSCs surviving GSKJ4 treatment. Conclusion: Our findings suggest that H3K27 methylation may have an inhibitory role in the maintenance of CSCs and that GSKJ4 may represent a novel class of CSC-targeting agents.*

Cancer stem cells (CSCs) are a small subpopulation of cancer cells that possess the capacity to self-renew themselves as undifferentiated cells with the potential to undergo differentiation into non-stem cancer cells, as well as the capacity to initiate and recapitulate tumors from which they are derived (1-3). In addition to such tumorigenic potential crucial to tumor development, CSCs are often endowed with resistance to conventional chemo- and radiotherapies, surviving treatments that would eradicate non-stem cancer cells. Thus, CSCs could play a pivotal role not only in the development but also recurrence of tumors even after seemingly successful treatments, and are therefore deemed as a major culprit in tumor recurrence and the poor prognosis of cancer (3-8). Understanding the mechanism underlying the cellular properties characteristic of CSCs, therefore, is now considered to be of utmost importance to achieve better management of human cancer.

A large body of evidence has indicated that CSCs have much in common with normal stem cells (9, 10). In this regard, it might be noteworthy that an 'open' chromatin state is a characteristic feature of pluripotent stem cells (11). Indeed, it was recently demonstrated that enrichment of trimethylation of histone H3 at lysine 27 (H3K27me3), a repressive chromatin mark, across the genome is associated with cellular differentiation not only *in vitro* but also *in vivo*, suggesting a possible role of this histone mark in the control of the stem cell state and differentiation of normal stem cells (12, 13). Interestingly, it is now becoming clear that the global level of H3K27me3 is reduced or lost in a variety of human cancer types. Most importantly, a number of independent studies have demonstrated that the global H3K27me3 level tends to be lower in less differentiated cancer and is significantly associated with worse prognosis (14-18). These observations suggest that the change in the global level of H3K27me3 may possibly have some role in determining the cellular fate of CSCs and hence the biological character of the cancer they give rise to. However, the role of this repressive histone mark in the maintenance and differentiation of CSCs still remains poorly understood.

GSKJ4 is a novel, selective inhibitor of the jumonji family histone demethylases JMJD3 and UTX, which are the H3K27me2/3-specific demethylases that catalyze the

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demethylation of H3K27me2/3 (19, 20). As such, GSKJ4 is anticipated to be a highly useful, promising tool for selectively modulating the cellular level of H3K27me3 for the purpose of exploring its role and significance in cellular functions (19). On the other hand, GSKJ4 has been drawing increasing attention as a novel anticancer agent since its anticancer effects were demonstrated against acute lymphoblastic leukemia and pediatric brainstem glioma in recent studies (21, 22). However, whereas the effect of GSKJ4 on the growth (including the cell cycle and apoptosis) of cancer cells was examined in those studies, its effect on CSCs, in particular on cellular properties unique to CSCs (*e.g.* self-renewal capacity, tumor-initiating capacity) has not been investigated and therefore remains totally unknown.

In this study, we tested the effect of GSKJ4 on CSCs using an ovarian CSC line as a model, along with the intention to explore the role and significance of H3K27me3 in CSCs. Ovarian cancer is a leading cause of female death in the world, in which CSCs have been considered to play a key role in tumor recurrence and hence its poor prognosis (23-27). Although we recently identified JNK inhibition as a novel approach to targeting ovarian CSCs (28), measures to target ovarian CSCs are still absolutely lacking. Herein we showed that GSKJ4 impairs not only the survival of ovarian CSCs but also their self-renewal and tumor-initiating capacity, quite effectively. Thus, our results highlight GSKJ4 as a promising CSC-targeting agent against ovarian cancer and possibly other human cancer types, and suggest at the same time that the global level of H3K27me3 may have a role in the maintenance and differentiation of CSCs as well as in normal stem cells.

Materials and Methods

Antibodies and reagents. Antibodies against sex-determining region Y-box 2 (SOX2) (#3579), Tir Na Nog (NANOG) (#4903), B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) (#6964), octamer-binding transcription factor 4A (OCT4A) (#2890), v-Myc avian myelocytomatosis viral oncogene homolog (c-MYC) (#9402), zinc finger E-box-binding homeobox 1 (ZEB1) (#3396), histone H3 (#4499), tri-methyl-histone H3 (Lys27) (#9733), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#5174) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-E-cadherin (sc-8426) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-CD133 (W6B3C1) was purchased from Miltenyi Biotech (Bergisch Gladbach, Germany). GSKJ4 was purchased from TOCRIS Bioscience (Bristol, UK) and was dissolved in dimethylsulfoxide (DMSO) to prepare a 10 mM stock solution.

Cell culture. The details of establishment of A2780 CSC, a CSC line derived from the human ovarian cancer cell line A2780, have been described elsewhere (28). The authenticity of A2780 CSCs as a cell line derived from A2780 was verified by genotyping of short tandem repeat (STR) loci (Bio-Synthesis, Inc., Lewisville, TX, USA) followed by comparison with the American Type Culture Collection (ATCC) STR database for Human Cell Lines. Unless otherwise indicated, A2780 CSCs were stably maintained and used for experiments under the monolayer stem cell culture condition, as previously described (28-30). In brief, cells were cultured on collagen-I-coated dishes (IWAKI, Tokyo, Japan) in a stem cell culture medium [Dulbecco's modified Eagle's 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 U/ml penicillin; and 100 μg/ml streptomycin]. In principle, the stem cell culture medium was changed every 3 days, and EGF and FGF2 were added to the culture medium every day. Normal human IMR90 fetal lung fibroblasts were obtained from the ATCC and maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. All IMR90 experiments were performed using cells with low passage number (<8).

Cytotoxicity assay. Viable and dead cells were identified by their ability and inability to exclude vital dyes, respectively (31, 32). Unless otherwise indicated, both adherent and non-adherent cells in the dishes were collected, and after centrifugation, resuspended in phosphate-buffered saline (PBS). The cell suspension was then mixed with an equal volume of PBS containing trypan blue (0.4% w/v, final concentration=0.2%) and examined under a phase-contrast microscope using a hemocytometer. The percentage of dead cells was defined as 100 × [number of dead cells/(the number of viable cells + dead cells)]. Alternatively, cells were incubated in situ with propidium iodide (PI, 1 μg/ml) and Hoechst 33342 (10 μg/ml) for 10 min at 37˚C in a CO_2 incubator to stain dead cells and the cell nuclei, respectively. Then the numbers of PI- and Hoechst-positive cells were scored under a fluorescence microscope (CKX41; Olympus, Tokyo, Japan), and the percentage of PI-positive cells (dead cells) against Hoechst-positive cells (total cells) was determined.

Flow cytometric analysis. Flow cytometric analysis was conducted as previously described (28, 33). Dissociated cells were washed with ice-cold PBS, fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature (RT), and washed again with PBS. The cells were blocked in FCM buffer [0.5% (w/v) bovine serum albumin and 0.1% (w/v) NaN3 in PBS] for 1 h, followed by three PBS rinses and further incubation with anti-CD133 antibody in the FCM buffer overnight at 4˚C, and then with Alexa Fluor® 488 goat anti-mouse IgG (Life Technologies, Carlsbad, CA, USA) for another 1 h at RT. Gating for single viable cells was established using forward scatter in the isotype control samples, which were used to establish a gate in the fluorescein isothiocyanate channel. Cells showing a signal for CD133 above the threshold established by the isotype control were deemed CD133-positive. All flow cytometric experiments were run on a 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).

Histone extraction. Histone extraction was performed using an acid extraction method (34, 35) with modifications, as described below. Cells were washed with chilled PBS once and lysed in hypotonic buffer [10 mM Hepes-KOH (pH 7.4), 10 mM KCl, and 0.5% NP-40] for 10 min on ice. The cell lysates were then agitated by pipetting and centrifuged at 4,000 × *g* for 10 min at 4˚C. After centrifugation, the

precipitates were washed once in hypotonic buffer, resuspended in 0.4 N HCl, and incubated overnight at 4˚C under shaking condition. The acid extracts were centrifuged at 14,000 × *g* for 10 min at 4˚C, and the supernatants were subsequently trichloroacetic acid-precipitated (final 33%) and centrifuged at 14,000 × *g* for 10 min at 4˚C again. The precipitates were washed with ice-cold acetone for removing acid and centrifuged at 14,000 × *g* for 10 min at 4˚C. The pellets were air-dried at RT and resuspended in urea buffer [2% sodium dodecyl sulfate (SDS), 10% glycerol, 60 mM Tris-HCl (pH 6.6), 4 M urea, and 0.5 mM dithiothreitol] prior to being subjected to immunoblot analysis.

Immunoblot analysis. Immunoblot analysis was conducted as previously described (28, 33). In brief, 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 (Sigma)]. After centrifugation for 10 min at 14,000 × *g* at 4˚C, the supernatants were recovered as cell lysates, and the protein concentration of the cell lysates was determined using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Cell lysates containing equal amounts of protein were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). The membrane was probed with a primary antibody and then with an appropriate horseradish peroxidase-conjugated secondary antibody, according to the protocol recommended by the manufacturer of each antibody. For E-cadherin detection, cells were washed with ice-cold PBS and lysed in Laemmli sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, and 0.004% bromophenol blue]. After boiling for 10 min, the lysates were subjected to the determination of GAPDH content by immunoblot analysis followed by densitometry using Image J software (http://imagej.nih.gov/ij/). Lysates containing an equal amount of GAPDH were then analyzed by immunoblotting for the expression of E-cadherin.

Sphere-formation assay. A sphere-formation assay was performed as previously described (28, 33). After dissociation into single cells, cells were examined for their viability and diluted in the stem cell culture medium. The cells were then seeded into non-coated 96-well plates such that each well-contained a single viable cell. On the next day of seeding, wells containing a single cell were marked under a phase-contrast microscope (wells containing only dead cell debris were excluded), and 1 week after seeding, the percentage of marked wells with a sphere relative to the total number of marked wells was determined.

Mouse xenograft study. A mouse xenograft study was conducted as previously described (28). After determination of the cellular viability, cells were suspended in 200 μl of PBS so that the cell suspension contained 2×10^6 viable cells. Then the cell suspension was injected into the peritoneal cavity of 5- to 8-week-old female BALB/cAJcl-nu/nu mice (CLEA Japan, Inc., Tokyo, Japan) to examine the tumor-initiating capacity of the cells (5 mice per group). The animal experiments conducted in this study were performed under the protocol approved by the Animal Research Committee of Yamagata University (no. 27106).

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

Results

GSKJ4 shows selective cytotoxicity against ovarian CSCs. To determine the range of concentrations of GSKJ4 that are not toxic to normal cells, we first treated IMR90 normal human fibroblasts with different concentrations of GSKJ4 and examined its effect on their growth and viability. Since the results indicated that IMR90 cell viability and growth remained unaffected at GSKJ4 concentrations up to 10 μM and 5 μM, respectively (Figure 1A), we selected 5 μM or lower for subsequent experiments. We then sought to evaluate the effects of GSKJ4 on CSCs using A2780 CSC, an ovarian CSC line established from A2780, as a model. Significantly, when A2780 CSCs were treated with GSKJ4 for 3 days, we found that GSKJ4 even as low as 1 μM was sufficient to inhibit the growth and viability of A2780 CSCs (Figure 1B). For confirmation, cell death was also analyzed by treating cell cultures *in situ* with a fluorescent vital dye, PI, instead of using trypan blue, enabling us to count the number of dead cells without losing dead cells during the cell harvesting procedure. The results obtained by this method similarly indicated that GSKJ4 induced cell death of A2780 CSCs at 0.5 μM and higher concentrations in a concentration-dependent manner (Figure 1C). Strikingly, over 80% of cells were dead when A2780 CSCs were treated with GSKJ4 at 2 μM. Thus the results suggested that GSKJ4 may have potent and selective cytotoxic acitivity against ovarian CSCs.

GSKJ4 inhibits the self-renewal capacity of ovarian CSCs. We next asked whether A2780 CSCs that had survived GSKJ4 treatment still retained their CSC properties. To this end, we first examined the effect of GSKJ4 treatment on the cell surface expression of CD133, a known CSC marker for various cancer types including ovarian CSCs (28, 36). As we have shown above, A2780 CSCs treated with 1 μM GSKJ4 for 3 days contained a significant fraction (approximately 40%) of dead cells (Figure 1). When we analyzed these cells by flow cytometry after gating-out dead cells, we observed a considerable reduction in the proportion of CD133-positive cells (Figure 2A). Consistent with the decrease in CD133 expression, GSKJ4 treatment also induced the down-regulation of the stem cell markers SOX2, NANOG, BMI1, OCT4A and up-regulation of an epithelial differentiation marker, E-cadherin (Figure 2B). Intriguingly, we also noticed a marked reduction after GSKJ4 treatment in the expression of ZEB1 and c-Myc, which are key transcription factors implicated in the control of the stem cell state (37, 38).

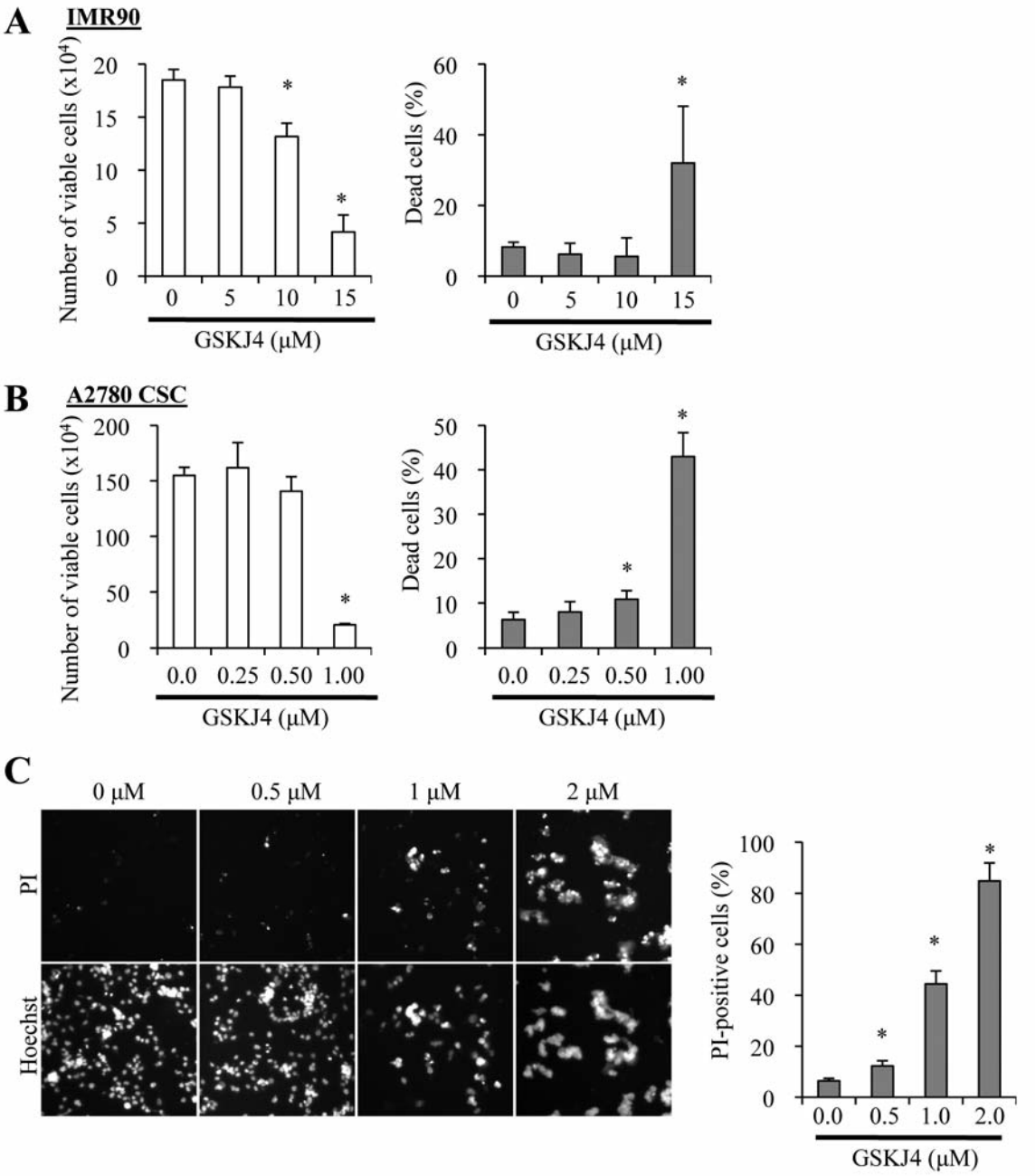

Figure 1. *GSKJ4 induces selective cytotoxicity against ovarian cancer stem cells (CSCs). IMR90 normal human fibroblasts (A) and A2780 CSCs (B) were treated with the indicated concentrations of GSKJ4 for 3 days. The number of viable cells (left panels) and the percentage of dead cells (right panels) were then determined using trypan blue as a vital dye. C: A2780 CSCs treated with the indicated concentrations of GSKJ4 were subjected to cell-death analysis using propidium iodide (PI) as a vital dye. Right, The percentage of PI-positive cells (dead cells) relative to Hoechst-positive cells (total cells) was determined. Left, Representative fluorescence images of PI- (upper rows) and Hoechst-positive cells (lower rows) are shown. Values in the graphs represent the mean + SD from three independent experiments. *p<0.05.*

Taken together, these results suggest that GSKJ4 treatment causes not only cell death but also loss of the self-renewal capacity of A2780 CSCs. To further substantiate whether GSKJ4 inhibits the self-renewal capacity of A2780 CSCs, we next examined the effect of GSKJ4 on their sphere-forming ability. The results of the sphere formation assay indicate that A2780 CSCs treated with 1 μM GSKJ4 for 3 days had markedly lost their ability to form spheres even in the

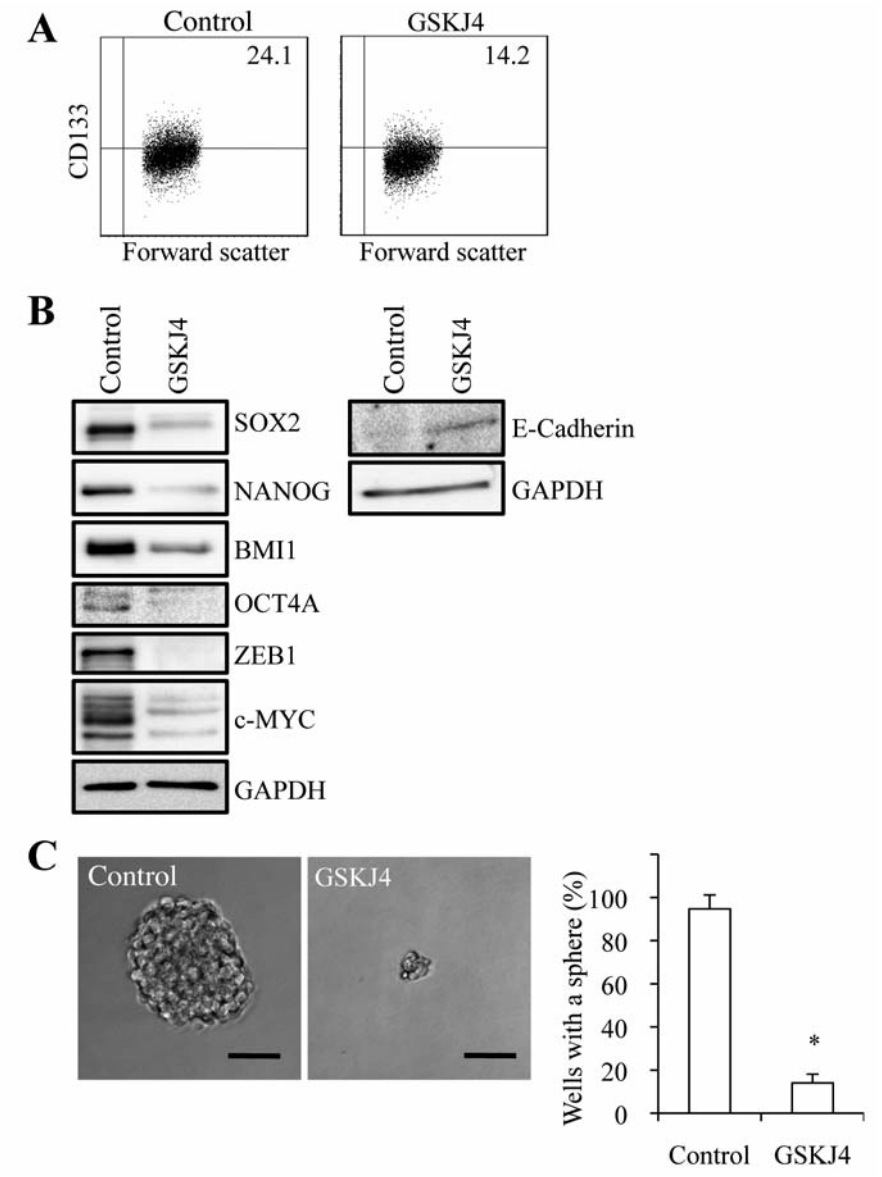

Figure 2. *GSKJ4 treatment causes loss of stem cell marker expression and sphere-forming ability in ovarian cancer stem cells (CSCs). A: A2780 CSCs cultured in the absence (Control) or presence of 1 μM GSKJ4 for 3 days were subjected to flow cytometric analysis for the cell surface expression of CD133. Representative flow cytometric plots together with the percentages of CD133-positive cells are shown. B: A2780 CSCs treated as in (A) were subjected to immunoblot analysis of the indicated proteins. C: A2780 CSCs treated as in (A) were subjected, after washing out the inhibitor, to the sphere-formation assay in the absence of GSKJ4. Right, The graph shows the percentage of wells in which a sphere formed from a single cell, and the data represent mean + SD from three independent experiments. *p<0.05. Left, Photographs of representative wells are shown. Scale bars, 50 μm. SOX2: Sex-determining region Y-box 2, NANOG: Tir Na Nog, BMI1: B-cell-specific Moloney murine leukemia virus integration site 1, OCT4A: octamer-binding transcription factor 4A, c-MYC: v-Myc avian myelocytomatosis viral oncogene homolog, ZEB1: zinc finger E-box-binding homeobox 1, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.*

absence of GSKJ4 (Figure 2C). To confirm that H3K27 demethylases were inhibited by GSKJ4 under the very experimental condition used herein, we also examined the global level of H3K27me3 in A2780 CSCs. Whereas the basal level of H3K27me3 was only barely detectable in control-treated A2780 CSCs, A2780 CSCs treated with 1 μM GSKJ4 showed a pronounced increase in the H3K27me3 level, suggesting that GSKJ4 inhibited the H3K27 demethylases quite effectively at this concentration (Figure 3). Taken together, the data suggest that GSKJ4 effectively

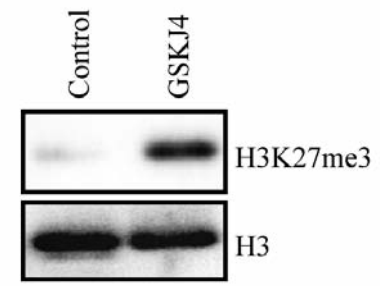

Figure 3. *Global increase in the trimethylation of histone H3 at lysine 27 (H3K27me3) in ovarian cancer stem cells treated with GSKJ4. A2780 CSCs were cultured either in the absence (Control) or presence of 1 μM GSKJ4 for 3 days. Then the cells were subjected to immunoblot analysis after histone extraction to determine the global level of H3K27me3 as well as the total H3 level.*

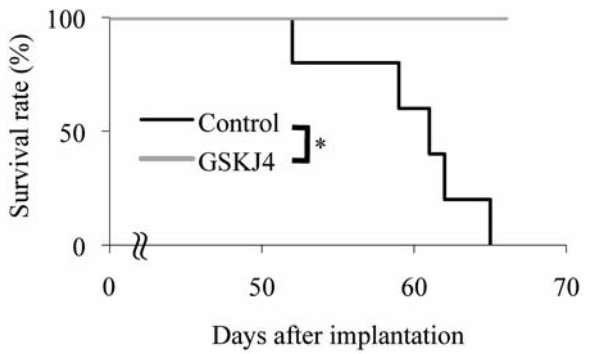

Figure 4. *The tumor-initiating capacity is lost in ovarian cancer stem cells surviving GSKJ4 treatment. A2780 CSCs were treated without (Control) or with GSKJ4 (1 μM) for 3 days, after which the cells were subjected to the determination of the cellular viability. An equal number (2×10^6) of viable cells were then implanted intraperitoneally into each nude mouse. Kaplan–Meier survival curves for the mice (n=5 for each group) are shown. *p<0.05.*

inhibits both the viability and self-renewal capacity of A2780 CSCs at a concentration at which it increases the global level of H3K27me3, most likely through inhibition of H3K27 demethylases.

GSKJ4 inhibits the tumor-initiating capacity of ovarian CSCs. Having shown that GSKJ4 not only induces cell death but also inhibits the self-renewal capacity of cells surviving GSKJ4 treatment, we further asked whether the tumor-initiating capacity, another key feature of CSCs, was lost similarly to the self-renewal capacity in A2780 CSCs surviving GSKJ4 treatment. To this end, we used the intraperitoneal xenograft model of ovarian cancer (28) and implanted intraperitoneally A2780 CSCs treated either with 1 μM GSKJ4 or control vehicle for 3 days into nude mice. To ascertain that the observed loss of the tumor-initiating capacity was not attributable to GSKJ4-induced cell death, we determined the viability of the cells after the 3-day GSKJ4 treatment and implanted an equal number of viable cells into nude mice. Around 1 month after implantation, all mice receiving control-treated cells started to show abdominal distension, which was overtly progressive and became prominent, and died by 65 days after implantation (Figure 4). In sharp contrast, mice implanted with GSKJ4-treated cells were healthy without any signs of intraperitoneal tumors at 2 months after implantation, except for one mouse that developed modest abdominal distension that was barely progressive. This far, the less progressive nature of the abdominal distension may suggest that the implanted tumor cells had limited tumor-initiating capacity, if any at all, and were incapable of perpetuating tumoral growth. All in all, the results indicated that GSKJ4 treatment impairs the tumor-initiating capacity of even A2780 CSCs that resist GSKJ4-induced cell death.

Discussion

With the increasing awareness that CSCs are a potential source of tumor recurrence after conventional cancer therapy (3-8), strategies to effectively target CSCs are now direly needed to realize long-term survival of cancer patients. Based on a number of evidence suggesting that the increase in the global level of H3K27me3 is positively associated with the differentiation of not only normal stem cells but also cancer cells (12-14), we hypothesized that interventions to elevate the cellular level of H3K27me3 might also have a differentiation-inducing effect on CSCs. We, therefore, in this study took advantage of GSKJ4, a selective inhibitor of the jumonji family histone demethylases, to promote methylation of H3K27 and tested its effect on ovarian CSCs. Rather unexpectedly, we first found that GSKJ4 induced cell death quite efficiently in ovarian CSCs at a concentration non-toxic to normal human fibroblasts. However, by examining cells that had survived GSKJ4 treatment for their stem cell characteristics, we successfully demonstrated that CSC properties were lost in surviving cells. Indeed, the surviving cells showed substantially reduced stem cell marker expression and sphere-forming ability, which suggests that their self-renewal capacity was impaired by GSKJ4. In parallel, the results of the xenograft analysis demonstrated that the tumor-initiating capacity was lost in ovarian CSCs surviving GSKJ4 treatment. Together, the results of the present study provide evidence that GSKJ4 effectively targets ovarian CSCs, eliminating ovarian CSCs through both cell death-dependent and -independent mechanisms, at least *in vitro*. To the best of our knowledge, this is the very first study to demonstrate the CSC-targeting effect of GSKJ4.

GSKJ4 was originally developed to selectively inhibit H3K27 demethylases and was first shown to be capable of

modulating the proinflammatory macrophage response, a process dependent on the demethylation of H3K27me3 at relevant gene promoters (19). Subsequently, two recent studies demonstrated that GSKJ4 has anticancer effects against acute lymphoblastic leukemia and pediatric brainstem glioma (21, 22). Although the effect of GSKJ4 on CSC-specific cellular properties was not investigated in those studies, notably, one of the studies showed the effect of GSKJ4 was specific to glioma cells that harbored the oncogenic K27M mutation of histone H3.3 and therefore had lower levels of H3K27me3 compared with those that did not harbor the mutation (21). The data, combined with the observations that the global H3K27me3 level is in general lower in stem cells than in differentiated non-stem cells (12-14), may lend support to an intriguing hypothesis that CSCs with low levels of H3K27me3 would be more sensitive to GSKJ4 than differentiated non-stem cancer cells with high levels of H3K27me3. To test this intriguing hypothesis, future studies are required to determine whether stem cells from cancer without K27M mutation of H3.3 are actually sensitive to GSKJ4 in contrast to their non-stem cell counterparts that are insensitive to GSKJ4.

The results of the present study also make a strong case that an increase in the overall level of H3K27me3 could have a causal role in inducing the loss of the stem cell characteristics in CSCs, although contribution of off-target effects of GSKJ4 cannot be excluded at this moment. It remains to be shown how the global increase in H3K27me3 induced by GSKJ4 culminates in CSC inhibition, but silencing of pluripotency genes such as *SOX2* and *OCT4* through methylation of H3K27 at their promoters may be among plausible explanations (39). It may also be worth noting here that, whereas GSKJ4 inhibits both JMJD3 and UTX, these two demethylases with a similar enzymatic function could have contrasting roles, with the former being essential for the initiation and maintenance of T-cell acute lymphoblastic leukemia and the latter functioning as a tumor suppressor and being frequently inactivated genetically in this disease (22). Future studies are therefore warranted to elucidate, by selectively inactivating each of JMJD3 and UTX, their respective roles in the maintenance of CSCs and whether or not the CSC-targeting effect of GSKJ4 depends on its inhibition of either or both of these demethylases.

In conclusion, using ovarian CSCs as a model, we have demonstrated for the first time in the present study that GSKJ4 can target CSCs, suggesting a critical role for H3K27 methylation in their maintenance and survival. Our findings, thus, provide an initial clue to explore the role of GSKJ4 as a potent CSC-targeting agent for ovarian cancer and other types of human cancer. Finally, future investigations of the CSC-targeting effect of GSKJ4 *in vivo* in preclinical animal studies are expected to provide data that will pave an avenue to develop epigenetic inhibitors as a new category of CSC-directed therapy.

Conflicts of Interest

The Authors declare no conflicts of interest.

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