Differential Contribution of ROS to Resveratrol-induced Cell Death and Loss of Self-renewal Capacity of Ovarian Cancer Stem Cells

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Abstract. *Background/Aim: Cancer stem cells (CSCs) are considered to contribute to the poor prognosis of ovarian cancer as a major cause of fatal recurrence. Identification of effective measures to eliminate ovarian CSCs through induction of cell death and/or loss of self-renewal capacity would, therefore, be key to successful management of ovarian cancer. Materials and Methods: The effects of resveratrol on the viability and self-renewal capacity of CSCs derived from A2780 human ovarian cancer cells were examined. The involvement of reactive oxygen species (ROS) was also investigated. Results: At a non-toxic to normal human fibroblasts concentration, resveratrol effectively killed ovarian CSCs independently of ROS, while ROS-dependently impaired the self-renewal capacity of ovarian CSCs that survived resveratrol treatment. Conclusion: Our findings not only shed light on a novel mechanism of action for resveratrol but also suggest that resveratrol, or its analogs, may be useful for CSC-directed therapy against ovarian cancer.*

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Resveratrol (3, 5, 4'- trihydroxystilbene), which was first isolated from the roots of white hellebore (*Veratrum grandiflorum O. Loes*) and later from the roots of *Polygonum Cuspidatum*, is a natural polyphenolic phytoalexin found in a variety of plant species, most typically in grapes, peanuts and berries (1, 2). Subsequent to the identification of resveratrol as an ingredient of red wine that might be responsible for its cardio-protective effects (called "French Paradox") (3), Jang and colleagues demonstrated a chemo-preventive effect of topically-applied resveratrol in skin carcinogenesis models in 1997 (4), which fueled intense research on resveratrol as an anti-cancer agent thereafter (1, 2, 5). Intriguingly, whereas a large body of *in vitro* evidence has suggested that resveratrol inhibits the proliferation and survival of various cancer cells, there is little *in vivo* evidence that resveratrol can serve as a viable treatment option once tumors are already formed (1, 5) pointing to the intriguing possibility that resveratrol may preferentially target the tumor-initiating process rather than the growth of bulk tumor cells. In line with such a possibility, cancer stem cells (CSCs), which are alternatively termed cancer-initiating cells because of their capacity to initiate tumors, are now emerging and drawing attention as a possible target of chemo-preventive agents (6, 7). Yet, currently, the information about the effects and the mechanisms of action of resveratrol on CSCs is still very limited (8-16).

Ovarian cancer is one of the most common and lethal gynecological malignancies being the fifth leading cause of cancer death in women worldwide (17). The poor prognosis of ovarian cancer is attributed mainly to frequent tumor recurrence, which occurs even after apparently successful

initial treatment and is usually fatal (18). Elimination of the CSC population of ovarian cancer, therefore, is now expected as a promising approach to improve survival of ovarian cancer patients through prevention of fatal recurrence for which CSCs are deemed responsible (19-21). However, although the effects of resveratrol or its analogs on ovarian cancer cells have been examined in some studies (22, 23), those of resveratrol on ovarian CSCs remain unexplored.

Here, in this study, we therefore investigated if and how resveratrol affects ovarian CSCs, using CSCs derived from the A2780 ovarian cancer cell line. The results suggested that resveratrol inhibits both the stem cell properties and viability of ovarian CSCs in reactive oxygen species (ROS)-dependent and -independent manners, respectively.

Materials and Methods

Antibodies and reagents. Anti-PARP (#9542), anti-cleaved-PARP (#9541), anti-caspase-3 (#9662), anti-cleaved-caspase-3 (#9661), anti-caspase-9 (#9502), anti-cleaved-caspase-9 (#9501), 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-CD133 (W6B3C1) was from Miltenyi Biotech (Bergisch Gladbach, Germany). Hydrogen peroxide solution (081-04215) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Resveratrol was purchased from Sigma and was dissolved in DMSO to prepare a 100 mM stock solution. N-acetylcysteine (NAC) was purchased from Sigma) and was dissolved in DMSO to prepare a 5 M stock solution. Z-VAD-FMK was purchase from Peptide Institute, Inc. (Osaka, Japan) and was dissolved in DMSO to prepare a 10 mM stock solution.

Cell culture. The details of the establishment of A2780 CSCs, CSCs derived from the human ovarian cancer cell line A2780, have been described (24). 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 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 (25, 26). In brief, cells were cultured on collagen-I-coated dishes (IWAKI, Tokyo, Japan) in 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 2 (FGF2) (Peprotech, Inc., Rocky Hill, NJ, USA), D-(+)-glucose (final concentration, 26.2 mM), 2 mM of L-glutamine (final concentration, 4.5 mM), 100 units/ml penicillin and 100 μg/ml streptomycin). 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 from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg/ml streptomycin. All IMR90 experiments were performed using low passage number (less than 9) cells. Throughout the study, the cell number was determined using a hemocytometer. Viable and dead cells were identified by their ability and inability to exclude vital dyes, respectively. Unless otherwise specified, both adherent and non-adherent cells in the dishes were collected and, after centrifugation, re-suspended in phosphate-buffer 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. Alternatively, cells were incubated *in situ* with propidium iodide (PI, 1 μg/ml) and Hoechst 33342 (10 μg/ml) for 15 minutes at 37˚C in the 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. The Hoechst staining was also used to determine the percentage of cells with condensed nuclei against total Hoechst-positive cells.

Sphere formation assay. After being dissociated into single cells, the cells were serially diluted in the stem cell culture medium and seeded into non-coated 96-well plates so that there would be a single cell in each well. Wells containing a single cell were marked on the next day and, 1 week after seeding, the percentage of marked wells with a sphere relative to the total number of marked wells was determined.

Immunoblot analysis. Cells were washed with ice-cold PBS and lysed in RIPA buffer (10 mM Tris-HCl (pH 7.4), 0.1% SDS, 1% sodium deoxycholate, 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 (Merck Millipore, Darmstadt, Germany)). 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 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. The membrane was probed with a primary antibody 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).

Flow cytometric analysis of CD133 expression. For the detection of CD133 expression, the dissociated cells were washed with ice-cold phosphate-buffered saline (PBS), fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature (RT) and washed again with PBS. The cells were then blocked in FACS buffer (0.5% (w/v) BSA, 0.1% (w/v) NaN_3 in PBS) for 30 min, followed by 3 PBS rinses and subsequently by incubation with the primary antibody in the FACS buffer for 1 h and then with the secondary antibody for another 1 h at RT. 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 (FITC) channel. Cells showing signal for CD133 above the gate established by the isotype control were deemed CD133-positive. All flow cytometric analysis experiments were run on FACSCanto™ II Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Detection and measurement of intracellular ROS. Cells were incubated with the stem cell culture medium containing 10 μM 2' ,7' -dichlorofluorescein diacetate (DCF-DA) for 10 min at 37˚C, washed twice with PBS and re-suspended in PBS. Care was taken to shield light during these procedures. Cells showing signal for DCF above the gate established by the isotype control were deemed ROS-positive. The cells were then subjected to flow cytometric analysis for quantification of the intensity of DCF fluorescence using a FACSCanto™ II Flow Cytometer (BD Biosciences).

Statistical analysis. Results are expressed as the means±standard deviation (SD) and differences were compared using the two-tailed Student's *t*-test. *p*-Values<0.05 were considered statistically significant and are indicated with asterisks in the Figures.

Results

Resveratrol inhibits the survival of ovarian CSCs in part via induction of apoptosis. To examine the effects of resveratrol on ovarian CSCs, we treated A2780 CSCs (CSCs established from the A2780 human ovarian cancer cell line (24)) with resveratrol at concentrations up to 50 μM, which has been reported to be non-toxic to normal human fibroblasts (27). Within this range of concentrations, resveratrol treatment reduced the number of viable A2780 CSCs in a concentration-dependent manner (Figure 1A), suggesting that resveratrol inhibited the survival and/or proliferation of A2780 CSCs. The results of the cell death assay showed that resveratrol induced substantial cell death of A2780 CSCs at 50 μM (Figure 1B). We also confirmed that the same concentration (50 μM) of resveratrol did not affect the growth of IMR90 normal human fibroblasts (Figure 1C). Thus, the results suggested that resveratrol may be selectively cytotoxic to ovarian CSCs. We next sought to determine the mode of A2780 CSC death induced by resveratrol. In line with previous studies reporting that resveratrol induces apoptosis of ovarian cancer cells (22, 28), we found that a significant proportion of resveratrol-induced A2780 CSC death was associated with typical chromatin condensation (Figure 2A). Biochemical analysis also demonstrated that resveratrol-induced cell death was accompanied by caspase activation as indicated by the increased expression of cleaved (active) caspases-3 and -9, as well as by the processing of the caspase substrate poly-ADP ribosyl polymerase (PARP) (Figure 2B). To determine whether the cell death was caspase-dependent apoptosis, we tested the effect of a pan-caspase inhibitor on resveratrol-induced A2780 CSC death. The pan-caspase inhibitor Z-VAD-FMK, which effectively inhibited the processing of PARP, inhibited resveratrol-induced cell death of A2780 CSCs, albeit partially (Figure 2C), suggesting that resveratrol-induced cell death was in part caspase-dependent.

Self-renewal capacity is impaired in A2780 CSCs surviving resveratrol treatment. We next asked whether or not A2780 CSCs that survived resveratrol treatment still retained stem cell properties. When the surviving fraction of resveratrol-treated A2780 CSCs was analyzed by flow cytometry for the cell surface expression of CD133, an ovarian CSC marker, we found that the proportion of CD133-positive cells was remarkably lower in resveratrol-treated cells compared with control-treated cells (Figure 3A). Consistent with the decrease in CD133 expression, resveratrol treatment also reduced the expression of stem cell transcription factors Sox2 and Nanog (Figure 3B). To determine whether the decreased expression of the stem cell markers occurred as a consequence of loss of the self-renewal capacity of surviving A2780 CSCs, we subjected equal numbers of viable cells from resveratrol- and control-treated samples to the sphere formation assay. The results of the sphere formation assay, which was conducted in the absence of resveratrol (*i.e*., after washout of resveratrol for the resveratrol-treated cells), clearly indicated that resveratrol-treated A2780 CSCs formed spheres much less efficiently than control-treated cells (Figure 3C) suggesting that cells that had survived resveratrol treatment had reduced self-renewal capacity.

Resveratrol increases the intracellular ROS level in A2780 CSCs, which may not nevertheless be essential for resveratrol-induced death of A2780 CSCs. The results thus far suggested that resveratrol induces cell death and inhibits the self-renewal capacity of A2780 CSCs at the same time. Since we have recently demonstrated that ROS play a key role in the negative regulation of CSC self-renewal, whereas ROS are a well-established inducer of cell death (29-33), we surmised that ROS might have a role in resveratrol-induced cell death and loss of the self-renewal capacity of A2780 CSCs. To test this idea, we first examined the effect of resveratrol treatment on the intracellular level of ROS in A2780 CSCs using 2′,7′-dichlorofluorescein diacetate (DCF-DA) as an indicator. The results indicated that resveratrol increased the intracellular ROS level in A2780 CSCs (Figure 4); ROS-positive cells increased up to nearly 50% at 24 h after resveratrol treatment, when resveratrol-induced cell death was evident (Figure 1B). We then examined whether ROS were required for resveratrol-induced cell death of A2780 CSCs. Whereas N-acetylcysteine (NAC) clearly, though partially, inhibited resveratrol-induced increase in intracellular ROS (Figure 4), unexpectedly, it neither inhibited resveratrol-induced cell death (Figure 5A) nor caspase activation (Figure 5B) suggesting that ROS may not necessarily be required for resveratrol-induced death of A2780 CSCs.

Critical role for ROS in resveratrol-induced loss of the self-renewal capacity of A2780 CSCs. We then asked if ROS could have a role in the loss of the self-renewal capacity of A2780 CSCs after resveratrol treatment. In

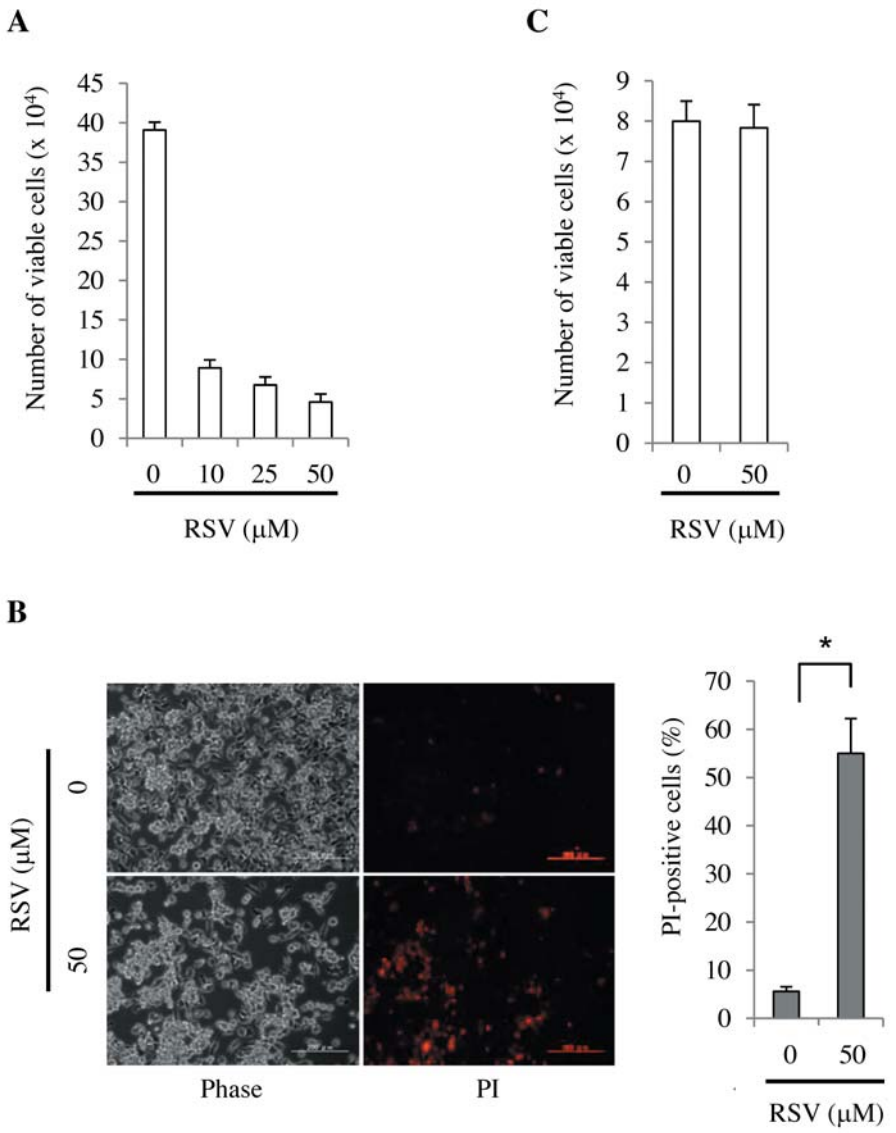

Figure 1. *Selective toxicity of resveratrol to ovarian cancer stem cells. (A) A2780 CSCs were cultured in the presence of the indicated concentrations of resveratrol (RSV) for 48 h, after which the number of viable cells was determined. Values represent means±SD from triplicate samples of a representative experiment repeated three times with similar results. (B) A2780 CSCs cultured in the presence of the indicated concentrations of RSV for 24 h were subjected to cell death assay using propidium iodide (PI) as a vital dye. Left, representative phase-contrast and PI fluorescence images are shown; Right, the percentage of PI-positive cells was determined. Values in the graph represent means±SD from triplicate samples of a representative experiment repeated three times with similar results. *P<0.05. (C) IMR90 normal human fibroblasts were cultured in the presence of the indicated concentrations of RSV for 48 h, after which the number of viable cells was determined. Values represent means±SD from triplicate samples of an experiment.*

contrast to resveratrol-induced cell death and caspase activation, which were not inhibited by NAC treatment as shown above, the decrease in CD133-positive cells (Figure 6A), as well as the expression of Sox2 and Nanog (Figure 6B), induced by resveratrol treatment was apparently inhibited by NAC. Consistent with these results, NAC at least partially blocked the resveratrol-induced loss of the sphere-forming ability of A2780 CSCs (Figure 6C) in support of the idea that ROS are required for the inhibition of the self-renewal capacity by resveratrol. We next went on to determine if increase in intracellular ROS is not only required but also sufficient to inhibit the stem cell properties of A2780 CSCs. To this end, we treated A2780 CSCs with hydrogen peroxide and examined its effect on

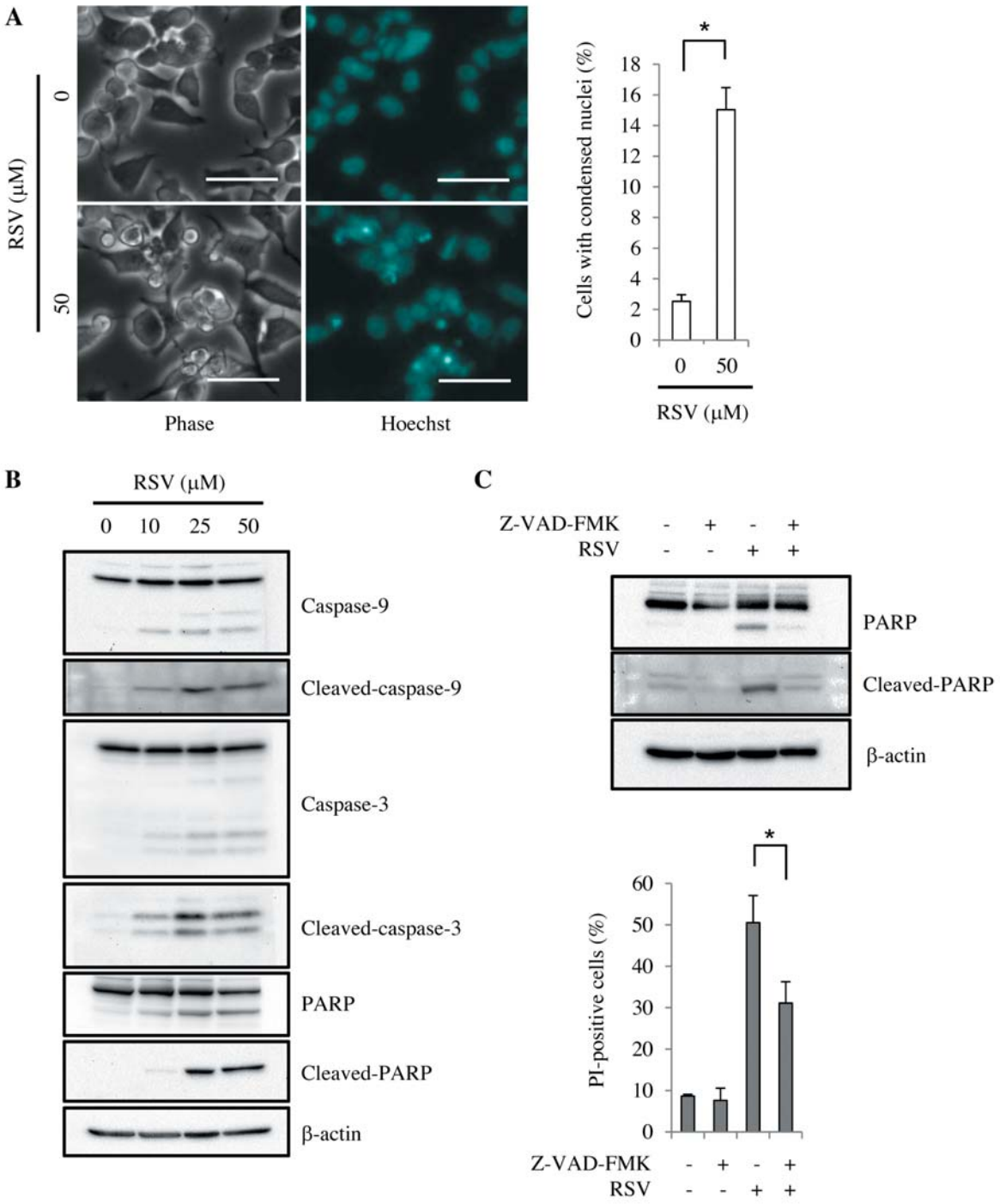

Figure 2. *Caspase activation and apoptosis induced by resveratrol in ovarian cancer stem cells. (A) A2780 CSCs cultured in the presence of the indicated concentrations of resveratrol (RSV) for 24 h were stained with Hoechst 33342 to examine their nuclear morphology. Left, representative phase-contrast and Hoechst fluorescence images are shown (scale bars, 50 μm); Right, the percentage of cells with condensed nuclei was determined. Values represent means±SD from triplicate samples of a representative experiment repeated three times with similar results. *p<0.05. (B) A2780 CSCs cultured in the presence of the indicated concentrations of RSV for 24 h were subjected to immunoblot analysis of the indicated proteins. (C) A2780 CSCs cultured in the absence or presence of 100 μM Z-VAD-FMK for 2 h were further treated with or without 50 μM RSV for 24 h. Then, the cells were subjected to immunoblot analysis of the indicated proteins (upper) or to cell death assay using propidium iodide (PI) as a vital dye (lower). The graph shows the percentage of PI-positive cells. Values represent means±SD from triplicate samples of a representative experiment repeated three times with similar results. *p<0.05.*

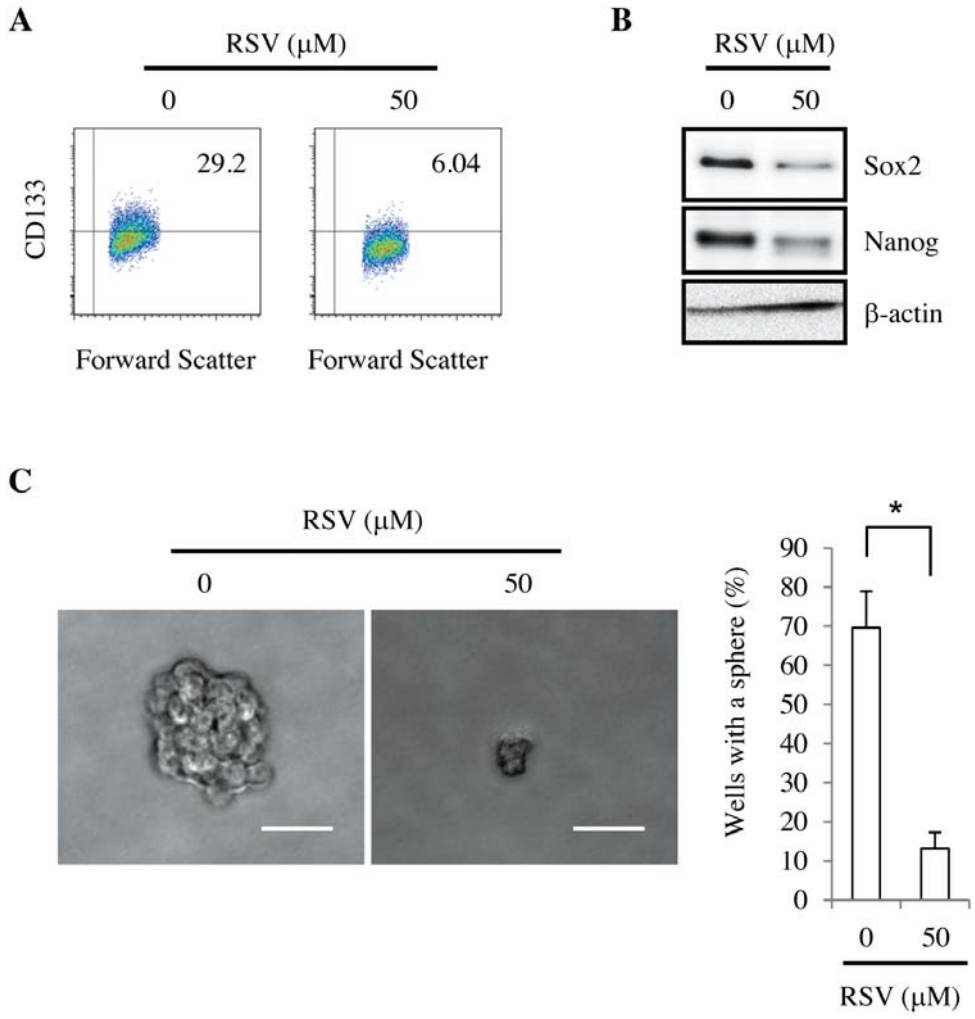

Figure 3. *Resveratrol treatment causes loss of the self-renewal capacity of ovarian cancer stem cells. (A) A2780 CSCs were cultured in the presence of the indicated concentrations of resveratrol (RSV) for 24 h and subjected to flow cytometric analysis for the cell surface expression of CD133. Representative flow cytometric plots are shown together with the percentages of CD133-positive cells. (B) A2780 CSCs cultured in the presence of the indicated concentrations of RSV for 36 h were subjected to immunoblot analysis of Sox2 and Nanog expression. (C) A2780 CSCs cultured in the presence of the indicated concentrations of RSV for 24 h were, after washout of RSV, subjected to the sphere formation assay in the absence of RSV. Left, phase-contrast photomicrographs of representative wells are shown (scale bars, 50 μm); Right, the percentage of wells in which a tumor sphere was formed from a single cell was determined. Values represent means±SD from triplicate samples of a representative experiment repeated three times with similar results. *p<0.05.*

the expression of stem cell markers. The results indicated that hydrogen peroxide, which increased intracellular ROS in a concentration-dependent manner (Figure 7A), decreased the expression of the stem cell markers, as well as the sphere-forming ability of A2780 CSCs accordingly (Figure 7B-D) suggesting that increase in intracellular ROS may be sufficient to inhibit the self-renewal capacity of A2780 CSCs. Altogether, the data suggested that ROS may be a key mediator of resveratrol-induced loss of the self-renewal capacity of A2780 CSCs.

Discussion

In the present study, we investigated the effects of resveratrol on ovarian CSCs using A2780 CSCs as a model and demonstrated that resveratrol not only induces ovarian CSC death effectively at a concentration that does not affect the survival and growth of normal human fibroblasts but also impairs the self-renewal capacity of ovarian CSCs that have survived resveratrol. Notably, although our data showed that caspase-dependent apoptosis was at least in

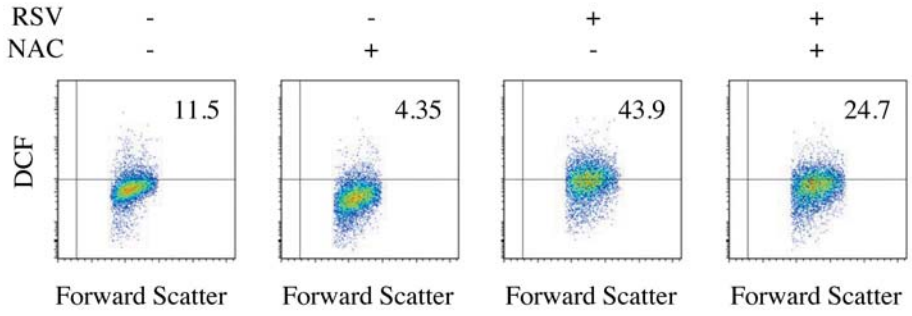

Figure 4. *Resveratrol increases the intracellular reactive oxygen species level in ovarian cancer stem cells. A2780 CSCs cultured in the absence or presence of 20 mM N-acetylcysteine (NAC) for 30 min and further treated with or without 50 μM resveratrol (RSV) for 24 h were stained with 2' ,7' -dichlorofluorescein diacetate (DCF-DA) and subjected to flow cytometric analysis to detect intracellular reactive oxygen species (ROS). Representative flow cytometric plots are shown together with the percentages of ROS-positive cells.*

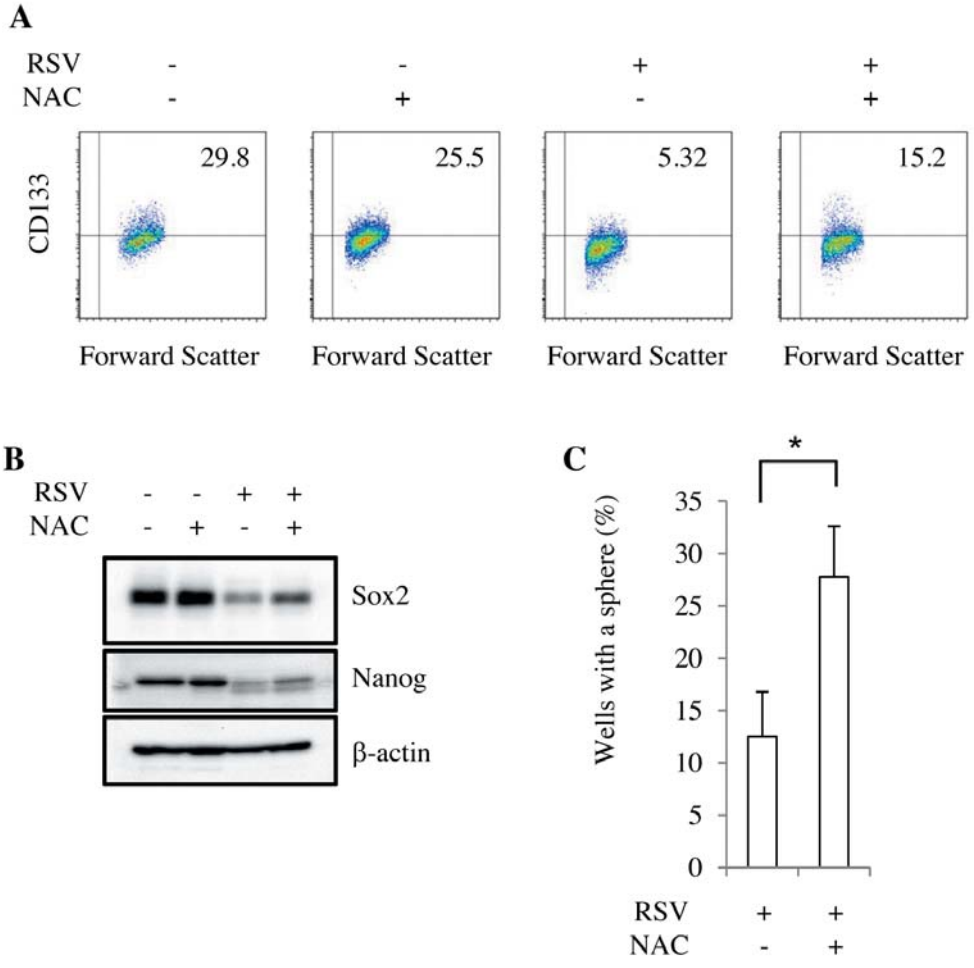

Figure 5. *Resveratrol-induced ovarian cancer stem cell death may not require reactive oxygen species. (A) A2780 CSCs cultured in the absence or presence of 20 mM N-acetylcysteine (NAC) for 30 min were further treated with or without 50 μM resveratrol (RSV) for 24 h. Then, the cells were subjected to cell death analysis using propidium iodide (PI). Left, representative phase-contrast and PI fluorescence images are shown; Right, the percentage of PI-positive cells was determined. Values represent means±SD from triplicate samples of a representative experiment repeated three times with similar results. *p<0.05. (B) A2780 CSCs treated as in (A) were subjected to immunoblot analysis of total and cleaved-PARP expression levels.*

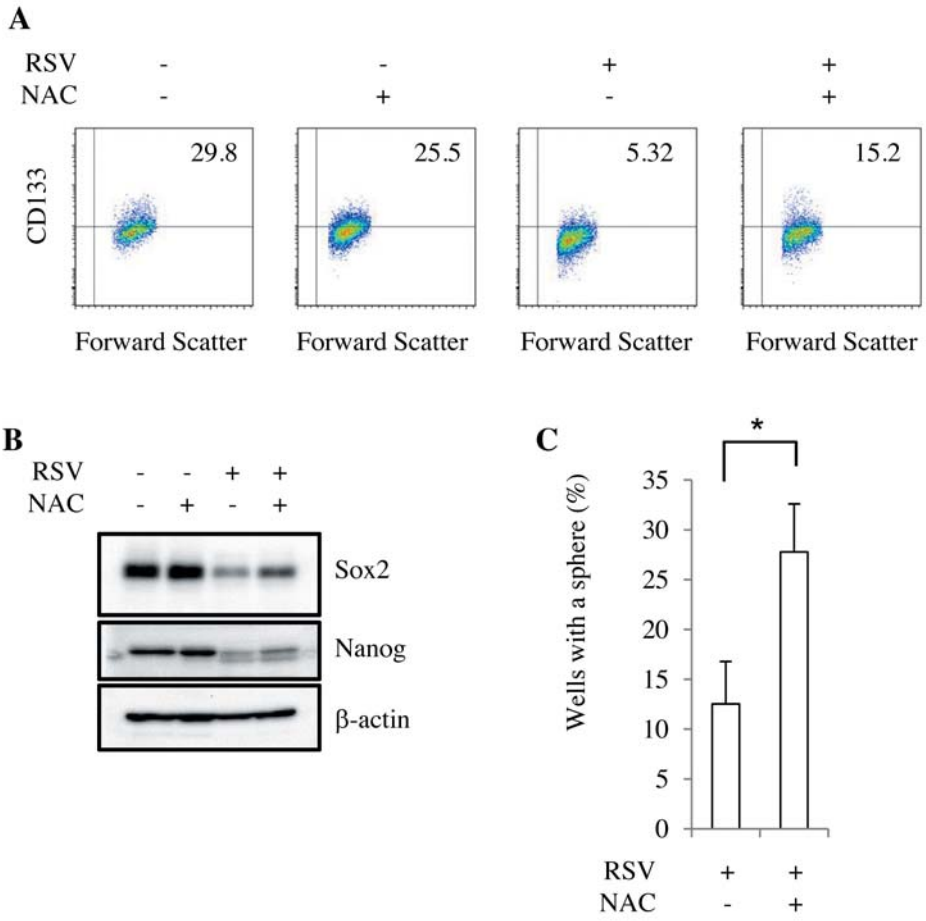

Figure 6. *Reactive oxygen species mediate resveratrol-induced loss of the self-renewal capacity of ovarian cancer stem cells. (A) A2780 CSCs cultured in the absence or presence of 20 mM N-acetylcysteine (NAC) for 30 min were further treated with or without 50 μM resveratrol (RSV) for 24 h. Then, the cells were subjected to flow cytometric analysis for cell surface expression of CD133. Representative flow cytometric plots are shown together with the percentages of CD133-positive cells. (B) A2780 CSCs cultured in the absence or presence of 20 mM NAC for 30 min and further treated with or without 50 μM RSV for 48 h were analyzed for the expression of Sox2 and Nanog by immunoblotting. (C) A2780 CSCs cultured in the absence or presence of 20 mM NAC for 30 min were further treated with or without 50 μM RSV for 24 h. Then, the cells were subjected, after washout of NAC and RSV, to the sphere formation assay in the absence of NAC and RSV. The graph shows the percentage of wells in which a tumor sphere was formed from a single cell. Values represent means±SD from triplicate samples of a representative experiment repeated three times with similar results. *p<0.05.*

part responsible for resveratrol-induced A2780 CSC death, the relatively small contribution of apoptosis to overall cell death (compare Figure1B with Figure 2A), as well as the partial inhibition of cell death, despite nearly complete caspase inhibition by the pan-caspase inhibitor (Figure 2C), implied that another non-apoptotic mechanism(s) of cell death was likely operative in resveratrol-induced ovarian CSC death. Because autophagic cell death is known as a representative form of non-apoptotic cell suicide (34-36) and was reportedly induced in A2780 cells by resveratrol (28), we suspected the possible involvement of autophagic cell death in resveratrol-induced CSC death. However, so far, our preliminary data indicate that resveratrol fails to increase the membrane-bound isoform of LC3 (LC3-II), an indicator of active autophagy (37), in A2780 CSCs (M.S. and M.O. unpublished observation). We, therefore, assume at this moment that other types of non-apoptotic cell death may be involved in resveratrol-induced ovarian CSC death. On the other hand, irrespective of the mode of cell death, the extensive cell death induced by resveratrol in this study made it more difficult to accurately assess the effect of resveratrol on the self-renewal capacity of A2780 CSCs. However, our flow cytometric analysis for CD133 expression conducted, after gating out dead cells, showed that CD133 expression was apparently decreased in the supposedly viable cell fraction

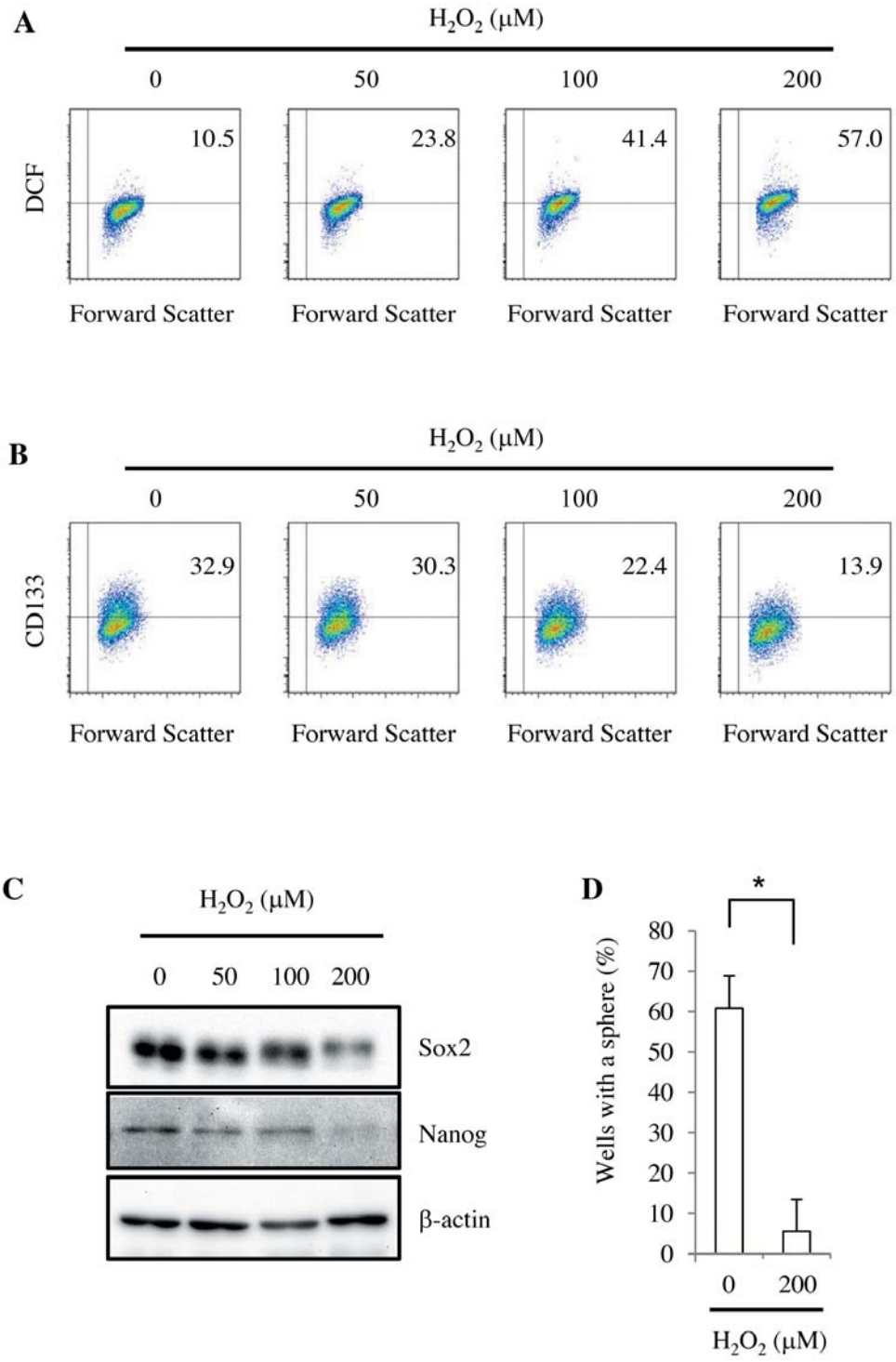

Figure 7. *Hydrogen peroxide treatment inhibits the self-renewal capacity of ovarian cancer stem cells. (A-C) A2780 CSCs cultured in the presence of the indicated concentrations of H_2O_2 for 30 min for (A) and 24 h for (B) and (C) were subjected to flow cytometry to analyze the intracellular level of reactive oxygen species after staining with 2' ,7' -dichlorofluorescein diacetate (DCF-DA) (A), to analyze the cell surface expression of CD133 (B) or were subjected to immunoblot analysis to examine the expression levels of Sox2 and Nanog (C). In (A) and (B), representative flow cytometric plots are shown together with the percentages of DCF- and CD133-positive cells, respectively. (D) A2780 CSCs cultured in the presence of the indicated concentrations of H_2O_2 for 24 h were subjected, after washout of H_2O_2, to the sphere formation assay in the absence of H_2O_2. The graph shows the percentage of wells in which a tumor sphere was formed from a single cell. Values represent means±SD from triplicate samples of a representative experiment repeated three times with similar results. *p<0.05.*

after resveratrol treatment (Figure 3A). Furthermore, the sphere-forming ability of resveratrol-treated A2780 CSCs was apparently reduced in our sphere formation assay in which we took care to determine the proportion of sphere-forming cells relative to "viable" cells instead of "total (=sum of viable and dead cells)" cells after resveratrol treatment (Figure 3C). We, therefore, consider that the impaired self-renewal capacity of A2780 CSCs after resveratrol treatment is unlikely a seeming consequence of resveratrol-induced cell death. Thus, our data suggest that both cell death and loss of self-renewal capacity were caused by resveratrol in ovarian CSCs; most likely independently of each other.

As an important finding of this study, we found that, in A2780 CSCs, resveratrol causes substantial increase in the intracellular ROS level and that it induces loss of self-renewal capacity and cell death in ROS-dependent and independent manners, respectively. Resveratrol has been widely recognized as a natural antioxidant (38, 39) but, notably, a couple of recent studies examining the effects of resveratrol on cancer cells, including CSCs, have demonstrated that resveratrol acts as a pro-oxidant, presumably in cell type- and context-dependent manners (15, 40, 41). Although the mechanism by which resveratrol increases intracellular ROS still remains unknown (15, 40, 41), our observation in conjunction with the previous reports further supports the emerging notion that resveratrol may act as a pro-oxidant in cancer cells. The observation that ROS were not required for resveratrol-induced cell death was rather unexpected given the prominent increase in intracellular ROS after resveratrol treatment of A2780 CSCs (Figure 4). However, it needs to be mentioned here that NAC could not completely suppress the resveratrol-induced increase in ROS in our study because of the toxicity associated with higher NAC concentrations, leaving the possibility that the residual increase in ROS was sufficient to mediate resveratrol-induced cell death. Nevertheless, our data clearly indicated that the partial inhibition of ROS increase was sufficient to block the loss of the self-renewal capacity of A2780 CSCs induced by resveratrol providing evidence that resveratrol-induced loss of self-renewal capacity is ROS-dependent (Figure 6). Such differential dependence of cell death and loss of self-renewal capacity on ROS strongly suggests that ROS may be differentially involved in resveratrol-initiated signaling pathways culminating in cell death and loss of self-renewal capacity.

So far, the effects of resveratrol on CSCs of some cancer types have been studied but its effects on ovarian CSCs have not yet been investigated leaving, thus, the underlying mechanism mostly unknown, with the exception that only fatty acid synthase and p53 have been implicated therein (8-16). Here, we demonstrated -for the first time- that resveratrol effectively kills ovarian CSCs and also inhibits their ability to self-renew in a ROS-dependent manner. Although we did not show how ROS inhibits the self-renewal capacity of ovarian CSCs in this study, it might be intriguing to speculate that ROS do so by activating p53 through DNA damage. In this regard, our preliminary data suggest that resveratrol increases the expression of the wild-type p53 protein in A2780 CSCs, in support of the idea. However, our data at the same time show that NAC fails to inhibit the resveratrol-induced up-regulation of p53 (M.S. and M.O., unpublished observation) suggesting that the effect of ROS on the self-renewal capacity of A2780 CSCs may not be p53-dependent. Apparently, future studies are warranted to elucidate the mechanism by which ROS affects the self-renewal capacity of CSCs of ovarian and other types of human cancer. Apart from the mechanism, our observation that ROS were necessary and sufficient for loss of the self-renewal capacity of ovarian CSCs has a significant implication in CSC biology as it strengthens the emerging idea that ROS play a pivotal role in the control of the self-renewal capacity of CSCs (32). Our results suggest that use of resveratrol is one viable measure to increase intracellular ROS in ovarian CSCs and presumably in CSCs of other cancer types but they also suggest that other measures to efficiently increase intracellular ROS in CSCs would also be useful, alone or in combination with resveratrol, as CSC-directed cancer therapies.

Ovarian cancer is a female malignancy with an unacceptably high mortality in which CSCs are highly implicated as a major culprit of fatal tumor recurrence. Elimination of CSCs, either by inducing cell death or by inhibiting self-renewal, is therefore considered essential to prevent recurrence and realize long-term survival and/or cure of ovarian cancer patients (19-21). Here, in the present study, we have demonstrated that resveratrol can target ovarian CSCs effectively *in vitro* through both induction of cell death and inhibition of self-renewal. Thus, the results of the present study suggest that resveratrol may be a promising agent to target ovarian CSCs and also provides a strong rationale to conduct preclinical studies to investigate the effects of resveratrol, and/or its analogs, on ovarian CSCs *in vivo*.

In conclusion, we demonstrated for the first time that resveratrol effectively eliminates ovarian CSCs *in vitro* through induction of cell death and inhibition of self-renewal capacity suggesting that resveratrol and its analogs could be candidate therapeutic agents for CSC-directed therapy against ovarian cancer. This demonstration that ROS play a role in resveratrol-induced loss of the self-renewal capacity of ovarian CSCs may underscore the importance of ROS as a regulator of CSC identity and also suggests that modulation of intracellular ROS might be an attractive approach to control CSCs.

Conflicts of Interest

The Authors declare no conflict of interest.

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