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Quiescence and Attenuated DNA Damage Response Promote Survival of Esophageal Cancer Stem Cells

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ABSTRACT

Accumulating evidence indicates cancer stem cells (CSCs) possess the capability to resist DNA-damage induced cell death, whereas the mechanism is largely unknown. Here we show that cell cycle status and DNA damage response (DDR) in CSCs probably contribute to their survival in genotoxic insults. In this study, we isolated esophageal cancer stem cells (ECSCs) from esophageal cancer cell line EC9706 by side-population (SP) phenotype through flow cytometry and found that ECSCs preferentially stay quiescent as compared to the non-ECSCs and are more resistant to DNA damage agents. Further study revealed that ECSCs express a lower level of EGFR, phosphoralated Stat3, and c-Myc, yet abnormally upregulated p27. More interestingly, different from non-ECSCs, when suffering DNA damage agents, ECSCs showed attenuated DDR, as well as declined DNA repair potential. These data indicated ECSCs probably employed an impaired DDR to handle severe genomic insults. Conclusively, we infer that the damage-resistance ability of ECSCs is likely attributed to their slow-cycling status and avoidance of apoptosis or senescence triggered by an excessive DDR. J. Cell. Biochem. 113: 3643–3652, 2012.

KEY WORDS: CANCER STEM CELLS; QUIESCENCE; DNA DAMAGE RESPONSE

Numerous studies have shown that a special subset of cells with stem-cell feature exists in many types of malignancies, referred as cancer stem cells (CSCs) or tumor initiating cells (TIC) [Reya et al., 2001; Dalerba et al., 2007; Gupta et al., 2009]. Rare CSCs retain the capability of resisting to radiation or chemotherapy, thereby explaining the almost-inevitable local recurrence after regular cancer treatment [Dean et al., 2005; Diehn et al., 2009]. So far, CSCs have already been isolated and identified in many kinds of tumors [Visvader and Lindeman, 2008]. Significant characteristics and critical regulating pathways in CSCs have been revealed [Bleau et al., 2009; Frank et al., 2010; Merchant and Matsui, 2010; Pannuti et al., 2010; Vermeulen et al., 2010; Seton-Rogers, 2011]. However, some fundamental questions are still not clearly answered, such as whether a strict hierarchy exists between CSCs and their offspring, whether CSCs are responsible for metastasis and relapse, and what mechanism makes rare CSCs survive radiation or chemotherapy.

Based on the recent knowledge, the resistance of CSCs to radiotherapy and chemotherapy is respectively attributed to lower ROS levels and ATP-binding cassette (ABC) drug transporters [Dean et al., 2005; Diehn et al., 2009; Vlashi et al., 2009]. In addition, other mechanisms adopted by normal adult stem cells to fulfill the damage-resistant potential might also apply to CSCs. Specifically, we propose that cell cycle regulation and DDR might contribute to the therapy-resistant ability of CSCs. Above all, adult stem cells in the majority of somatic tissues are largely in quiescent state, which is believed as an essential mechanism to resist DNA damage [Orford

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and Scadden, 2008; Li and Clevers, 2010]. Besides, adult stem cells have special DNA damage response (DDR) and repair pathways to retain their resistance to DNA-damage induced cell death compared with their progenies [Mandal et al., 2011]. For instance, short duration and attenuated activation of tumor suppressor p53 determine the survival of stem cell suffering DNA damage insults [Blanpain et al., 2011]. However, the existence of dormant CSCs has not been directly demonstrated, and the cell cycle status of CSCs in homeostasis is still controversial. Al-Hajj et al. [2003] did not find significant differences in cell cycle distribution between tumorigenic and nontumorigenic breast cancer cells, whereas Roesch et al. [2010] noted JARID1B^{+} cells in human melanoma were more tumorigenic and cycled more slowly than JARID1B^{-} cells. Additionally, the understanding of DDR in CSCs is also very limited. Bao et al. [2006] showed that glioma stem cells (GSCs) preferentially activated DDR thereby increasing DNA repair capacity, which contributed to their radioresistance.

In this study, we investigated cell cycle regulation and DDR in CSCs. We utilized esophageal cancer cell line EC9706 as research model, and identified esophageal cancer stem cells (ECSCs) by side-population (SP) phenotype. Previous studies showed that CSCs could be enriched through SP phenotype in many tumor cell lines as well as fresh cancer specimens [Wang et al., 2007; Zhou et al., 2007; Bleau et al., 2009]. Recently, ECSCs have been identified and isolated from esophageal cancer cell lines and clinical specimens according to the SP phenotype [Huang et al., 2009; Li et al., 2011]. These SP cells have a differential gene expression profile, special regulating pathways, better therapy-resistant capacity and stronger tumorigenicity in vivo compared to non-SP cells [Huang et al., 2009; Li et al., 2011]. In consistent with these studies, we found ECSCs were more resistant to ultraviolet radiation (UV) or cisplatin. More importantly, we showed that ECSCs are more quiescent than non-ECSC cells, which was associated with a lower level of phosphoralated Stat3 and c-Myc, yet higher level of p27. Moreover, when exposed to DNA damage agents, ECSCs exhibited impaired induction of p53 and declined G_1 checkpoint arrest, as well as attenuated DNA repair potential, as compared to non-ECSCs. Taken together, these data indicated that the relatively quiescent state and attenuated DDR in ECSCs possibly contributed to their radioresistance and chemoresistance.

MATERIALS AND METHODS

CELL CULTURE AND ISOLATION

Esophageal cancer cell line EC9706 was grown in RPMI 1640 (Invitrogen, Carlsbad, CA) culture supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin, at 37°C humidified atmosphere with 5% CO_2. Before isolation, cells were detached with trypsin and resuspended in RPMI 1640 containing 2% fetal bovine serum at the concentration of 1×10^6 cells/ml. Then the cells were incubated in 37°C with Hoechst 33342 (Sigma–Aldrich, St. Louis, MO) at 5 μg/ml for 90 min either alone or following a pre-treatment of verapamil (Sigma–Aldrich) at 50 μg/ml for 15 min. After staining, the cells were washed and maintained at 4°C for flow cytometry analysis and sorting (Beckman Coulter, Miami, FL).

COLONY FORMING EFFICIENCY (CFE) ASSAY

Freshly sorted SP and non-SP cells were plated with the same amount (1,000 cells/well), and were exposed to UV (1 mJ/cm^2). After being cultured normally for about 14 days when most colonies reached 30–50 cells, colonies were stained with crystal violet and counted. The CFE assay was calculated by dividing the number of colonies in the groups with irradiation by the number of colonies in the control groups and expressed as a percentage.

CELL CYCLE ANALYSIS AND BrdU LABEL ASSAY

Freshly isolated cells were straightly fixed with 70% ice-cold ethanol overnight at 4°C, and stained with propidium iodide (PI) (Sigma–Aldrich) and RNase for an expected time before flow cytometry analysis. For BrdU incorporation, cells were co-cultured with BrdU (BD PharMingen, San Diego, California) for 12 h, and washed twice with PBS. These cells were then stained with Hoechst 33342 and isolated for SP and non-SP cells, which were then subjected to BrdU label analysis with FACS.

INDUCTION OF DNA DAMAGE AND SYNCHRONIZATION

To induce DNA damage, cells were washed with PBS and exposed to UV (200 μw/cm^2) or cisplatin (20 μg/ml) for an expected time, and then washed with PBS twice and cultured normally for subsequent research. For synchronization, the cells were co-cultured with 2 μg/ml nocodazol (Sigma–Aldrich) for 15 h at 37°C, and then harvested for cell cycle analysis.

PRIMERS

The ABCG2 primers were forward primer: TGG GCA TCA TGG TGT ATA GAC G and reverse primer: GGG ACA GGT ATG TGA AAA GCA G. The β-actin primers were forward primer: GCA CCA CAC CTT CTA CAA TG and reverse primer: TGC TTG CTG ATC CAC ATC TG. The GAPDH primers were forward primer: TGT TGC CAT CAA TGA CCC CTT and reverse primer: CTC CAC GAC GTA CTC AGC G.

WESTERN BLOT AND ANTIBODY

After the total protein was extracted from individual SP and non-SP cells, immunoblot analysis was performed as described [Ji et al., 2007]. The cyclin D1 (1:500), cyclin E (1:300), cyclin B (1:500), cyclin A (1:500), p53 (1:1,000), Stat3 (1:500), p27 (1:500), and c-Myc (1:500) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The EGFR (1:500), Phospho-Stat3 (1:1,000), Gadd45a (1:500), and Phospho-Rb (1:500) antibody was obtained from Cell Signaling Technology (Beverly, MA). And, β-actin (1:5,000) antibody was bought from Sigma–Aldrich.

IMMUNOFLUORESCENT STAINING

Cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature and then permeabilized with 0.3% Triton-X-100 for 20 min at room temperature. Then, cells were blocked with 1% BSA for 1 h, followed by incubating with primary antibody (anti-phospho-H2AX, Abcam, Cambridge, UK, 1:500) overnight at 4°C. After that, cells were washed and incubated with fluorescence-labeled secondary antibody (1:400) for 30 min at room temperature. Cells were then washed and counterstained with DAPI

(1 μg/ml; Sigma–Aldrich). The coverslips were mounted onto glass slides and observed with a fluorescent microscope.

STATISTICAL ANALYSIS

Quantified data are presented as mean ± SD significance testing was performed with Student's *t*-test and *P* values of <0.05 were considered statistically significant.

RESULTS

ENRICHMENT OF CSCs IN ESOPHAGEAL CANCER CELL LINE THROUGH SP PHENOTYPE

Firstly, we isolated SP cells in an esophageal cancer cell line EC9706. As expected, incubation in the presence of 50 μg/ml verapamil before labeling Hoechst 33342 could abolish the SP phenotype, helping us to ensure the SP fraction (Fig. 1A,B). Besides, to confirm the SP isolation, we also tested the expression of ABCG2, an ABC transporter contributing to SP phenotype through pumping Hoechst 33342 out of the cells (Fig. 1C). Real-time PCR analysis showed that SP cells have a much higher level of ABCG2 expression than non-SP cells (Fig. 1D). Taken together, these data indicated that SP fraction was isolated successfully.

ECSCs ARE MORE RESISTANT TO DNA DAMAGE AGENTS

How CSCs response to genotoxic insults determines the outcomes of majority of cancer treatment. To evaluate the sensitivity of ECSCs to DNA damage agents, SP and non-SP cells were respectively subjected to UV. In the colony-forming assay, the SP cells showed a significantly higher colony forming efficiency than non-SP cells after UV attack (Fig. 2A,B), indicating ECSCs are more resistant to UV induced DNA damage. No conspicuous difference was observed in control groups between SP and non-SP cells. Moreover, MTT assay was employed to test the resistance of these two populations to cisplatin induced cell death. The results showed that ECSCs are more resistant to cisplatin-induced cell death than non-ECSCs (Fig. 2C). To substantiate these observations, EC9706 cells were incubated with different dose of cisplatin for 30 h, followed by SP ratio analysis through flow cytometry. The results showed an increase in SP ratio in the groups treated with 2 μg/ml cisplatin, which should be attributed to either a decrease in the amount of non-SP cells or an increase of SP cells (Fig. 2D,E). Taken together, ECSCs in EC9706 have a stronger ability to resist cell death induced by UV or cisplatin.

ECSCs ARE MORE QUIESCENT COMPARED TO NON-ECSCs

It is well accepted that the DNA-damage induced signaling pathways and consequences are largely dependent on the cell cycle status [Mandal et al., 2011]. To decipher the mechanism underlying the resistance of ECSCs to DNA-damage induced cell death, we analyzed the cell cycle kinetics of ECSCs in the steady state. SP and non-SP cells were stained with PI immediately after isolation, followed by the analysis of cell cycle distribution through flow cytometry. There was 73.1 ± 3.9% of SP cells in G_0/G_1 phase, while

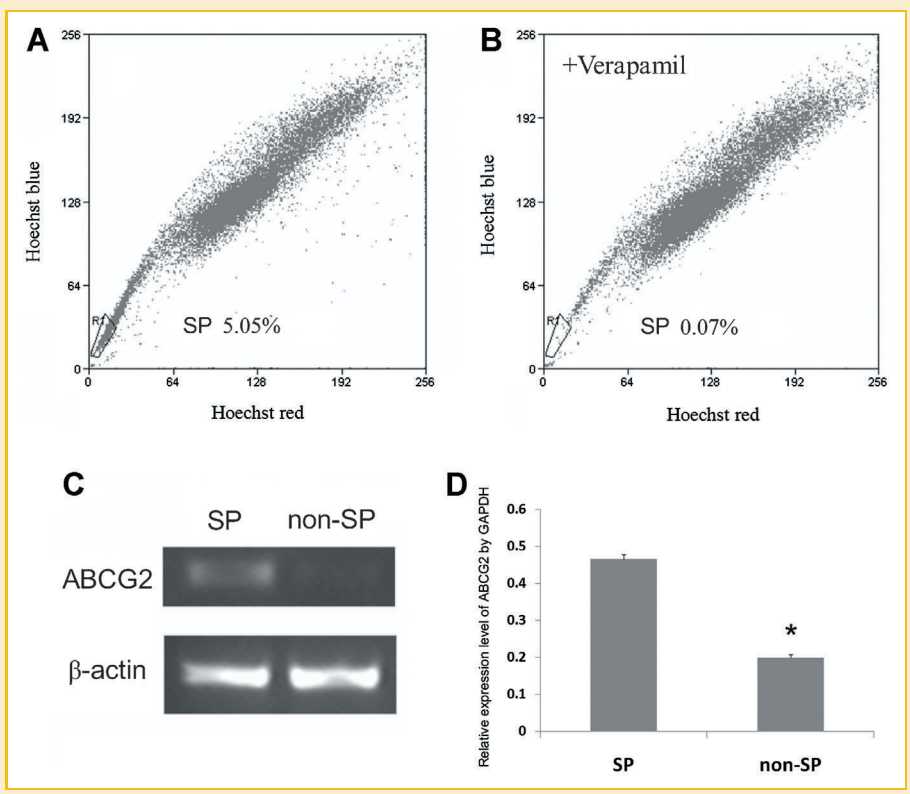

Fig. 1. Identification of SP population in esophageal cancer cell line (A) Hoechst 33342 dye exclusion assay on EC9706 identifies a SP fraction, accounting for about 5% in a steady state. B: Incubation with verapamil (50–100 μg/ml) for 20 min before staining abolishes the SP population. C: RT-PCR and (D) qRT-PCR show a higher level of ABCG2 mRNA in SP fraction as compared to non-SP population. n = 3; $^*P<0.01$.

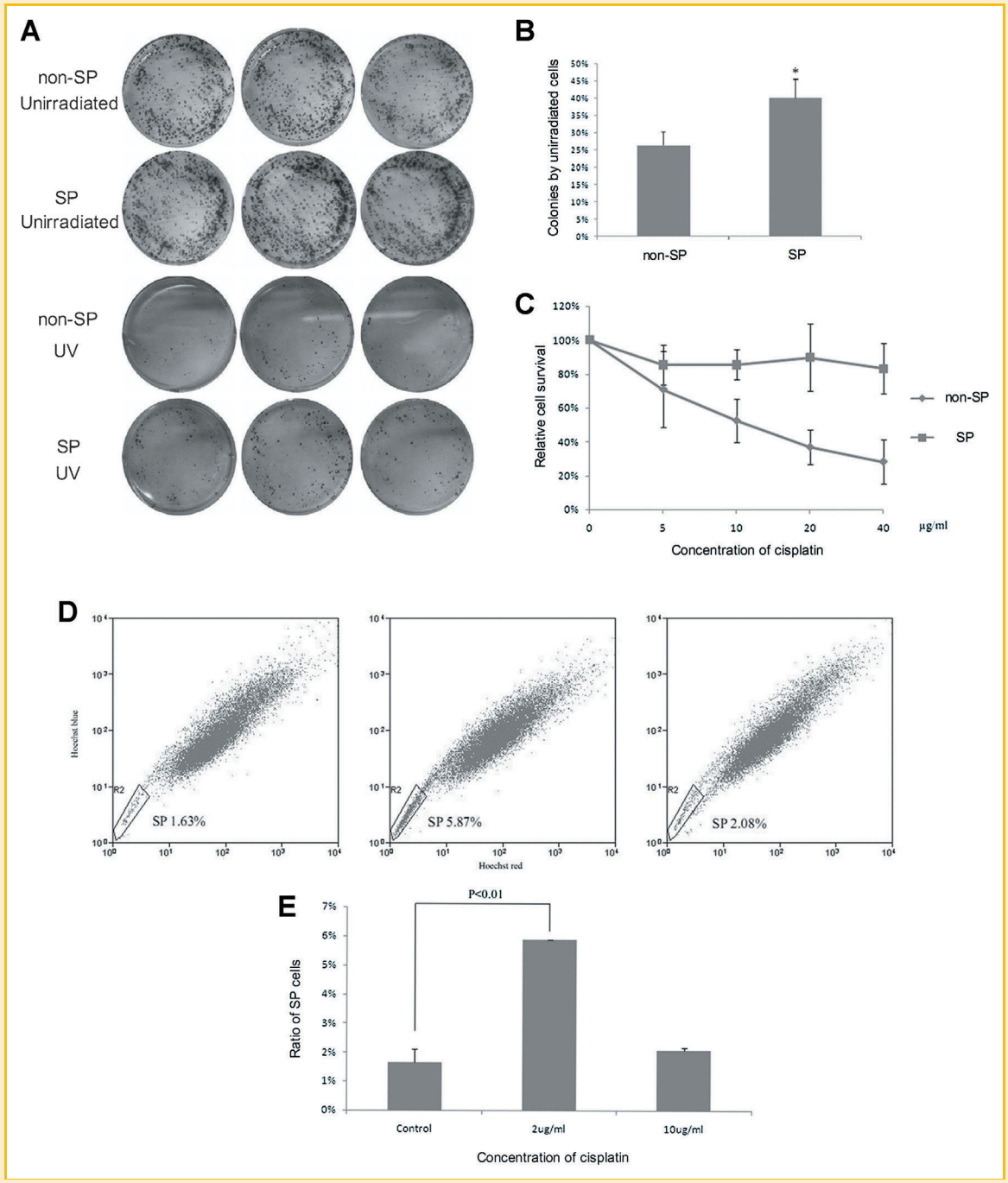

Fig. 2. ECSCs are more resistant to DNA damage agents. A: Colony forming assays are performed on SP and non-SP cells with the same number after UV (1 mJ/cm^2). The colonies are counted 2 weeks after irradiation. B: Colony forming efficiency of SP and non-SP cells in (A) identifies SP cells more resistant to radiation. n = 3; $^*P < 0.01$. (C) MTT assays show more SP cells survive treatment with cisplatin. (D) EC9706 is co-cultured with cisplatin (2 or 10 μg/ml) for 30 h, followed by SP-ratio analysis. (E) Statistical analysis on (D) shows an increase in SP ratio, induced by incubation with cisplatin (2 μg/ml). n = 3.

the non-SP cells in G_0/G_1 phase only accounted for 54.7 ± 3.5% (Fig. 3A,B). To further dissect G_0 phase, we used Pyronin Y (PY) and Hoechst 33342 to co-stain the total population before isolation and analyzed the proportion of G_0 phase in SP and non-SP cells. Flow cytometry analysis showed the proportion of the PY^{low} fraction in SP population was 55.43 ± 1.32%, while that in non-SP population was 19.02 ± 0.38% (Fig. 3C,D). Furthermore, to learn about the cell cycle status in a relatively long-term period, we employed BrdU incorporation assay to investigate the cell cycle kinetics. EC9706 was cultured with BrdU for 12 h until SP and non-SP cells were sorted out; the proportions of BrdU-labeled cells was analyzed respectively through flow cytometry. In EC9706, 31.35% of non-SP cells incorporated BrdU, while in SP fraction, the cells that labeled BrdU accounted for only 15.98% (Fig. 3E). Additionally, cyclin D, E,

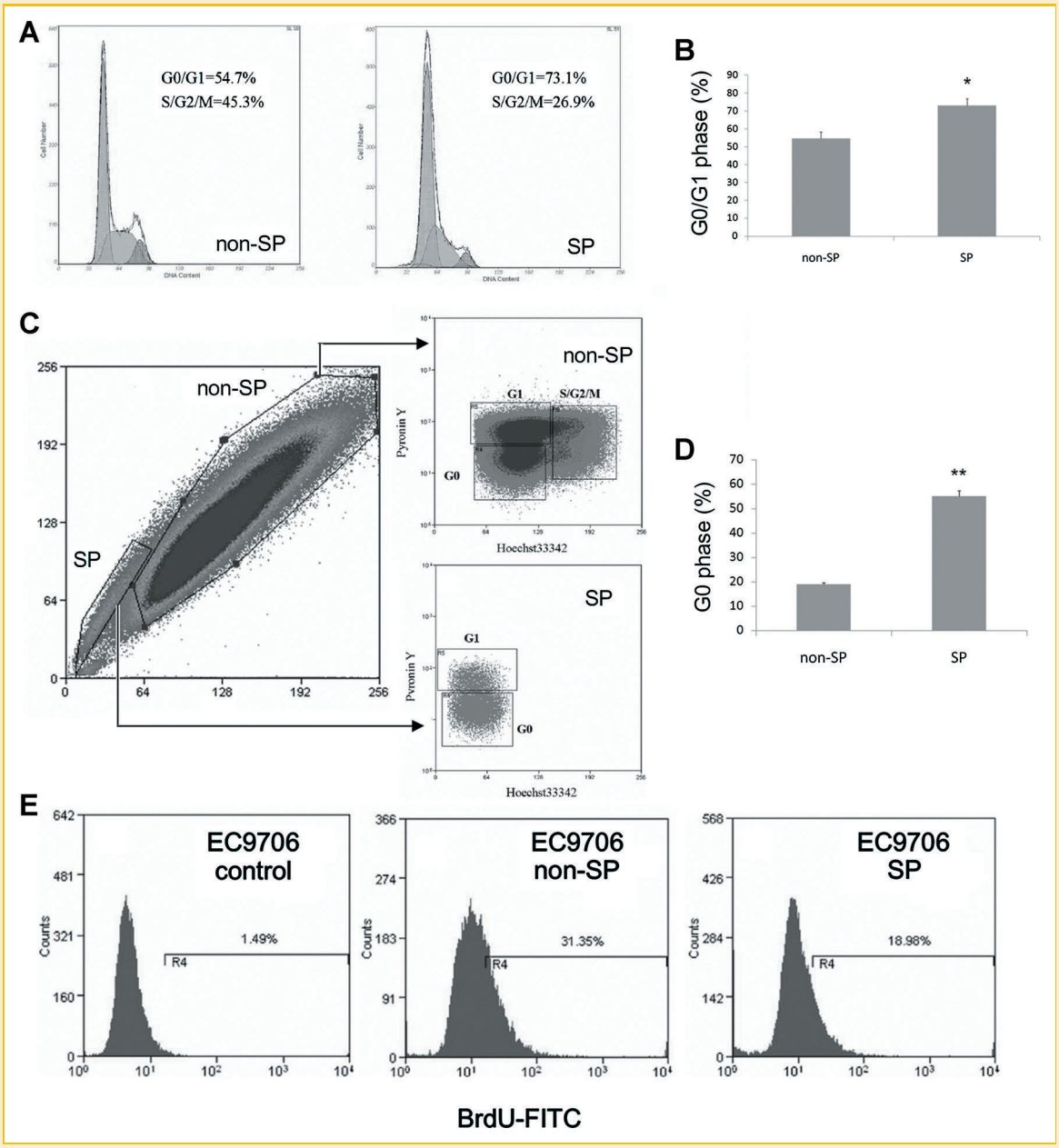

Fig. 3. ECSCs harbor more quiescent cells in unperturbed homeostasis. A: Cell cycle distribution is analyzed through staining with PI on SP and non-SP freshly isolated and fixed with 70% ethanol. B: Flow cytometry analysis on (A) shows that a larger fraction of SP cells stay in quiescent state. n = 3; $^{*}P < 0.01$. C: Co-staining with PY and Hoechst 33342 identified SP population harbors more G_0 cells with a relatively low PY staining. D: Statistical analysis on the proportions of PY^{low} populations in SP and non-SP fraction shows more cells in SP population are in G_0 phase. n = 3; $^{**}P < 0.001$. E: BrdU is labeled in EC9706 for 12 h followed by isolating SP and non-SP cells, which are subsequently subjected to detection of BrdU. Results show SP cells are in a relatively slow-cycling state.

A, and B were detected in freshly sorted SP and non-SP cells by Western blot (Fig. 4A). Cyclin D1 is the first cyclin produced in early G_1 phase, while Cyclin E is highly expressed in late G_1 phase and contributes to G_1/S transition. In freshly sorted SP cells in EC9706, we found Cyclin D1 increased and Cyclin E decreased compared to non-SP cells. Besides, Cyclin A and Cyclin B, which are highly expressed in S and G_2M phases, were found to decrease in SP cells. The results showed more cells in SP population were in early G_1 phase compared to non-SP cells. Taken together, these data indicates that ECSCs harbor more quiescent cells than non-ECSCs.

EGFR/STAT3/c-Myc/p27 PATHWAY MIGHT CONTRIBUTE TO THE QUIESCENCE OF ECSCs

To probe the key molecules controlling the quiescence of ECSCs, we detected the expression of several cell-cycle related proteins respectively from freshly isolated non-SP and SP cells. Immunoblot analysis showed a significantly declined expression of EGFR,

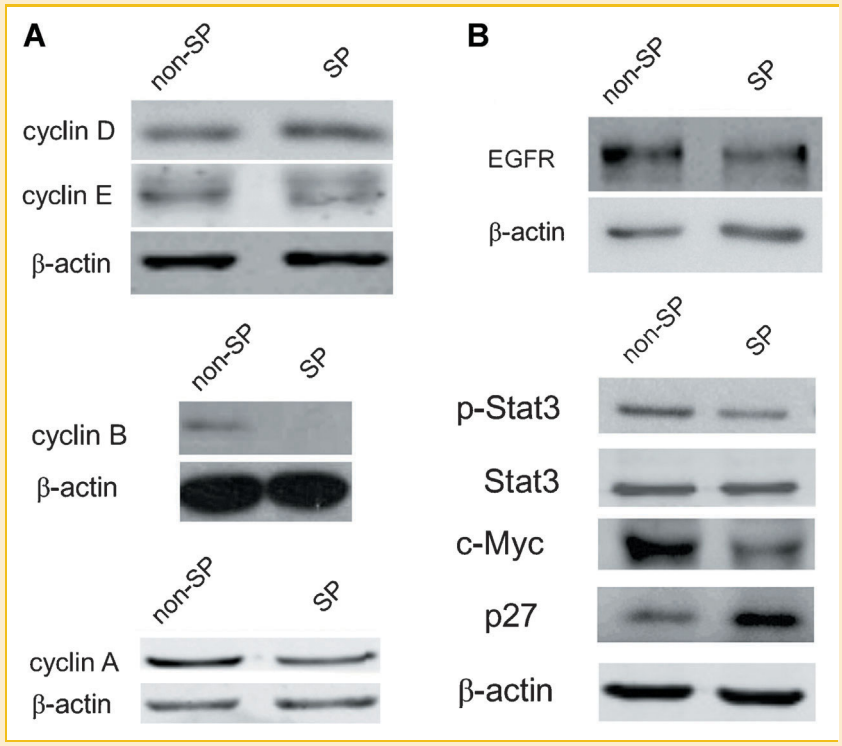

Fig. 4. The expression of several cycling-relative proteins in the steady state. A: Cyclin D, E, A, and B were detected in freshly sorted SP and non-SP cells by Western blot. The results showed more cells in SP population were in early G_1 phase compared to non-SP cells. B: EGFR was tested in freshly sorted SP and non-SP cells by Western blot. A lower expression level of suggests a decreased growth stimulating signals in SP population. Immunoblot analysis shows freshly sorted SP cells express a lower level of EGFR, phosphorylated Stat3 and c-Myc but a higher p27 than non-SP cells in the steady state.

phosphorylated Stat3 (Ser705), and c-Myc as well as a markedly enhanced expression level of p27 in SP cells (Fig. 4B). A lower expression level of EGFR suggests a decreased growth stimulating signals in SP population. Previous reports evidenced that Stat3 promotes cell cycle progression through positively regulating c-Myc in cancer and embryonic stem cells [Kiuchi et al., 1999; Amin et al., 2004; Cartwright et al., 2005]. Besides, c-Myc was verified to negatively regulate p27 [Yang et al., 2001], a cell-cycle inhibitor maintaining quiescence in adult stem cells [Zou et al., 2011]. All these evidence suggests a possible EGFR/Stat3/c-Myc/p27 pathway might contribute to the maintenance of the quiescent SP cells. Accordingly, we hypothesize that the high level of p27 in SP cells might be the reason for the quiescent cell cycle state, which is likely to be inhibited by the activated EGFR/Stat3/c-Myc pathway in non-SP cells.

ECSCs EXHIBIT AN ATTENUATED DDR WHEN SUFFERING DNA DAMAGE AGENTS

Furthermore, c-Myc was reported to promote induction of p53 and to be required for DNA-damage induced cell cycle arrest and apoptosis [Alarcon et al., 1996; Rogulski et al., 2005; Guerra et al., 2010]. Considering the different expression levels of c-Myc, we tested p53 induction in SP and non-SP cells after exposure to UV. Immunoblot analysis showed that, compared with a conspicuous activation of p53 in non-SP cells, SP cells presented impaired induction of p53 (Fig. 5A). Besides, we detected expression of Rb and

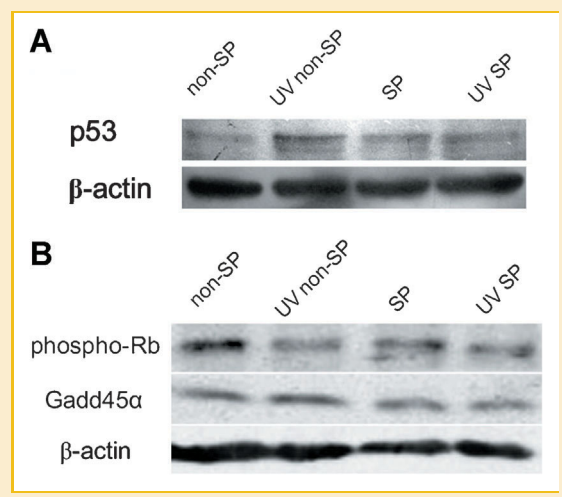

Fig. 5. The expression of several cell cycle checkpoint proteins after UV-attack. A: The expression of p53 at 6 h post-exposure to UV (3 mJ/cm^2) is tested by immunoblot, which shows p53 is not induced by UV in SP cells. B: The expression of phospho-Rb and Gadd45a were tested by Western blot 7 h post-UV attack (6 mJ/cm^2). The UV induced decrease of phosphor-Rb and increase of Gadd45a were much more noticeable in non-SP population than SP population.

Gadd45a, which respectively controls G_1 and G_2M checkpoint, after DNA damage. There was a much less reduction of phospho-Rb and an impaired induction of Gadd45a in SP population compared to non-SP cells (Fig. 5B). Taken together, these results indicated an attenuated DNA damage induced cell cycle checkpoint in ECSCs.

To test the cell cycle arrest in ECSCs with impaired induction of p53, we subjected SP and non-SP cells to UV or cisplatin and examined the ability of them in inducing cell cycle arrest after DNA damage. For the purpose of testing G_1 arrest excluding the impact of cells that reenter G_1 from M phase, we exploited nocodazol to block cells at G_2/M phase, so that G_1 arrest after DNA damage attack must be totally attributed to the G_1 checkpoint. SP and non-SP cells were subjected to UV or cisplatin, followed by co-culture with nocodazol for another 14 h. The analyses of the cell cycle distribution showed that, cells blocked at G_1 phase caused by DNA damage in SP population were much fewer than those in the non-SP population (Fig. 6A,B), indicating that ECSCs showed an attenuated induction of G1 cell cycle arrest compared to non-ECSCs upon treatment with DNA damage agent. Similarly, we used aphidicolin, which can block cells at early S phase, to test G_2/M arrest excluding the influence of G_1 checkpoint. The results showed that the G_2/M arrest in SP population was also impaired after DNA damage (Supplementary Fig. 1).

Given the cell cycle arrest is recognized as a premise of DNA repair, we wondered how the DNA repair potential could be impacted by the attenuated cell cycle arrest. We further evaluated the DNA repair ability of SP and non-SP cells, respectively. We stained phosphorylated gamma-H2AX, a sensitive indicator of DNA damage and repair, after exposing to 3 mJ/cm^2 of UV. The gamma-H2AX foci increased with no significant difference in SP and non-SP cells during the first 20 h post-UV. However, from the 20th hour post-UV, gamma-H2AX foci in non-SP cells decreased in spite of

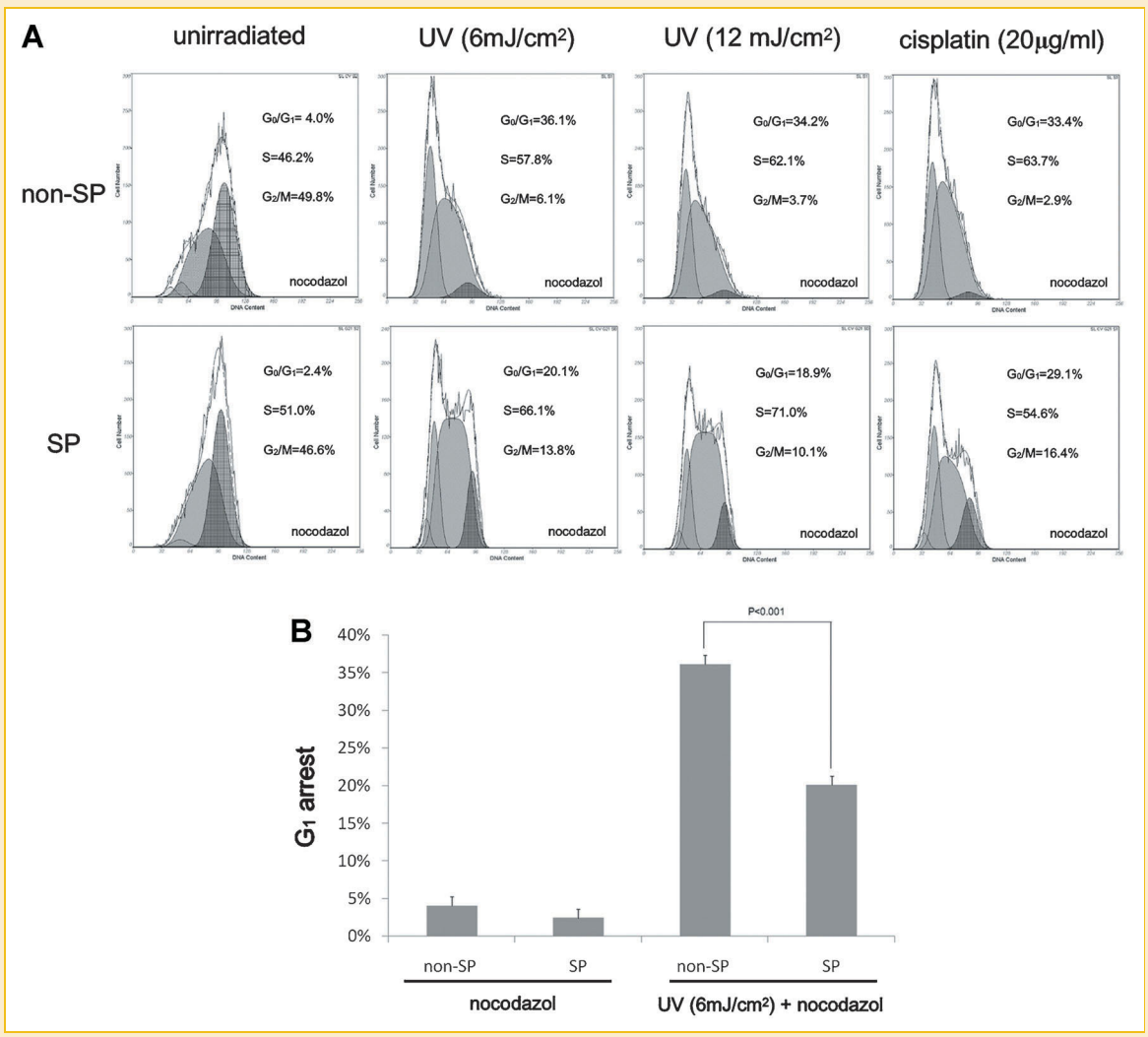

Fig. 6. ECSCs induce an attenuated cell cycle arrest to cope with DNA damage insults (A) The cell cycle arrest of SP and non-SP cells in DNA damage situation is analyzed by flow cytometry. Freshly isolated cells are subjected to DNA damage agents (6 mJ/cm^2, 12 mJ/cm^2 UV or 20 μg/ml cisplatin for 12 h) and then incubated with nocodazol (2 μg/ml) for 14 h to examine the G_1 cell cycle arrest. Flow cytometry analysis shows that the proportion of cells in G_1/G_0 phase in SP population is smaller as compared to non-SP population. B: Statistic results of (A) show fewer cells in SP fraction blocked at G_1 checkpoint 26 h after UV (6 mJ/cm^2) attack. $n = 3$; $P < 0.001$.

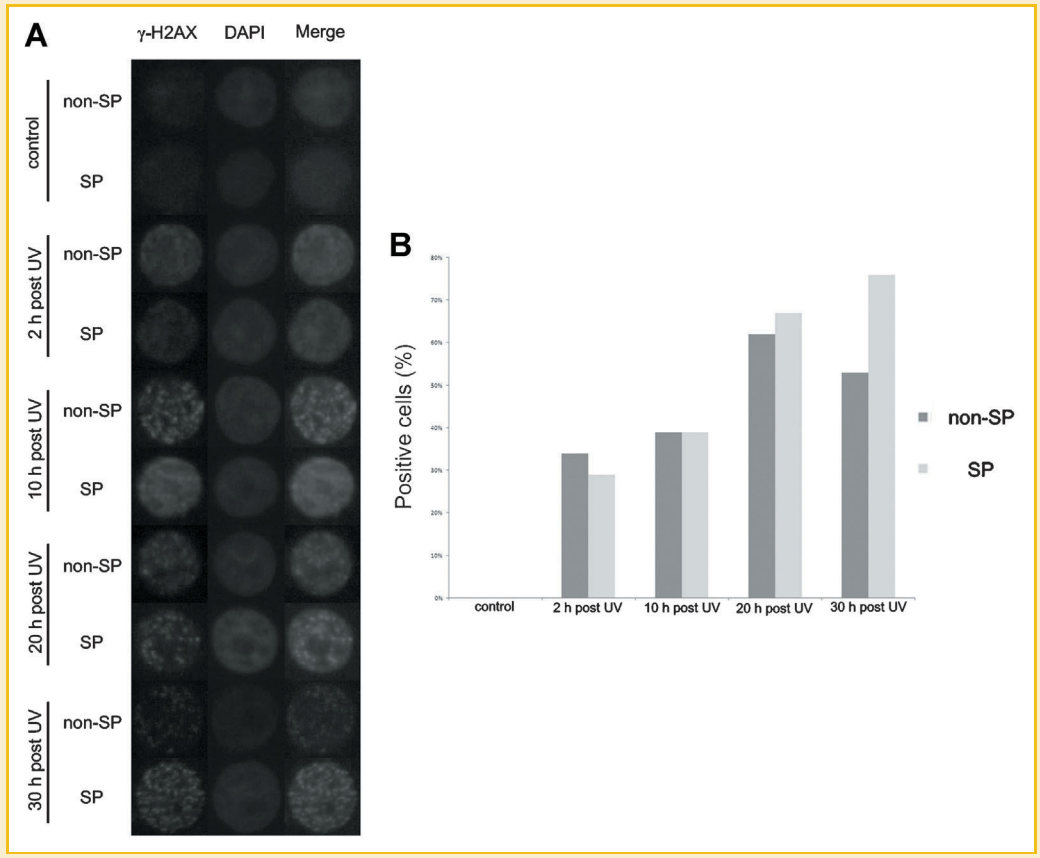

Fig. 7. Declined DNA repair potential in ECSCs (A) SP and non-SP cells are exposed to UV (3 mJ/cm^2), and cells are fixed and immunofluorescent staining of γ-H2AX nuclear foci is performed after a certain hours of recovery. B: Percentage of positive cells (>5 foci) in SP and non-SP population is calculated at different time points post-UV. In non-SP population, percentage of cells with damaged DNA decreases after 20th hour post-UV, while the damage in SP cells is still increasing.

still increasing in SP cells (Fig. 7A,B). These data suggested that the increased survival of ECSCs due to defective induction of p53 and attenuated cell cycle arrest could eventually provoke an accumulation of DNA damage, which might be deleterious in the long run.

DISCUSSION

Therapy resistance is demonstrated as one of the main features in CSCs, though the mechanisms are still elusive [Dean et al., 2005]. We investigated the cell cycle status in unperturbed homeostasis and DDR under genotoxic insults to reveal the mechanisms of drug and radiation resistance in ECSCs. In our study, we found ECSCs (SP cells) in EC9706 are more resistant to genotoxic insults and more quiescent than non-ECSCs. Slow-cycling status is widely considered to be essential for cancer cells to avoid insults, because of the declined DNA duplication and metabolism. Besides, quiescence in ECSCs probably plays a central role in maintaining the ECSC pool to sustain a tumor, since many evidence suggest proliferation induces differentiation and exhaustion of stem cells and increases accumulation of mutations in mostly adult tissues [Fleming et al., 2008; Orford and Scadden, 2008]. Accordingly, it is logical to recognize the slow-cycling status contributes to the DNA damage resistance of ECSCs, at least partially.

Additionally, we found ECSCs induce an attenuated DDR when exposed to UV or cisplatin, which probably contributes to their survival in genotoxic insults. Many evidence support attenuated duration and strength of DDR in adult stem cells, especially the deduced activation of p53, facilitate their survival in DNA damage attack [Blanpain et al., 2011; Mandal et al., 2011]. Under genotoxic insults, p53 is recognized to arrest cell cycle progression to repair damaged DNA. If the lesion is irreparable, p53 induces apoptosis or senescence to maintain genome stability [Levine, 1997; Kastan and Bartek, 2004]. Prolonged activation of p53 is believed to provoke cell death or aging [Elmore, 2007; Vazquez et al., 2008], which might explain why the non-ECSCs with stronger activation of p53 are more sensitive to UV in our study. Furthermore, prolonged cell cycle arrest could also induce apoptosis or senescence to eliminate the damaged cells [Campisi and d'Adda di Fagagna, 2007]. Therefore, impaired induction of p53 and reduced cell cycle arrest might be a protecting mechanism for ECSCs to survive severe DNA damage insults. This behavior of ECSCs contributes to sustain the existence of a tumor at the expense of a stable genetic background, leaving access to variation, which might explain why many drugs become inefficient after tumor recurrence.

In previous study, Bao et al. [2006] reported that GSCs exhibited a preferential activation of the DDR and thereby increased DNA repair capacity to resist IR-induced cell death. Nevertheless, our data

revealed an impaired induction of p53 and attenuated cell cycle arrest in ECSCs under UV or cisplatin attack. We raise possible reasons to explain the paradox as below. (1) Different tissues were investigated. In somatic stem cell research, evidence suggests variant patterns, and outcomes of DDR exist in various tissues [Blanpain et al., 2011]. Therefore, ECSCs and GSCs may employ distinct mechanisms to response to DNA damage. (2) The degree of DNA damage was different. We have compared the injury outcomes of cells exposed to different agents and found the damage in our study was much more severe than Bao et al. used. For this reason, we suppose the distinct pattern of DDR in ECSCs might be due to the damage in our study was too severe to be restored. Facing the irreparable damage, the only way to survive is to avoid excessive and persistent activation of DDR. This condition might better simulate the clinical treatment. (3) Different parts of DDR were focused on. Bao et al. did not examine the p53 and cell cycle arrest, which were emphasis in our study. Activation of p53 and induction of cell cycle arrest are both essential parts of DDR that directly determine the outcomes.

In this study, we employed SP phenotype to identify and isolate ECSCs, considering the stability of the CSC surface markers is questioned recently. Several studies evidenced that the stem-like cells in tumors seemly had no stable connection between the phenotype and the cell surface markers [Quintana et al., 2010; Roesch et al., 2010; Sharma et al., 2010]. Since SP phenotype is widely recognized as effective enrichment of stem cells by functional feature, we focused on the SP cells in order to provide a better insight into the functional characteristics of ECSCs.

In conclusion, we investigated the cell cycle regulation and DDR of ECSCs in the steady state and severe DNA-damage situation. We found ECSCs employ different mechanisms in regulating cell cycle status and handling DNA damage as compared to non-ECSCs. ECSCs are quiescent in the steady stage, but undergo attenuated cell cycle arrest under severe DNA damage stresses. Our findings provide a better insight into the mechanisms of CSCs resistance to radiotherapy and chemotherapy, therefore leading to new therapeutic targets and better treatment strategies of malignancies.

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