Aberrantly elevated redox sensing factor Nrf2 promotes cancer stem cell survival via enhanced transcriptional regulation of ABCG2 and Bcl-2/Bmi-1 genes

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Abstract. The presence of cancer stem cells in cervical cancer stem cells (CSCs) is important in the prevention of therapy failure and tumor recurrence. Upregulation of transcriptional regulation of redox sensing factor Nrf2 leads to the overexpression of drug efflux proteins such as ABCG2 in cancer stem cells and thus results in cancer treatment failure and cancer relapse. In the present study, we purified approximately 3.1% of cervical CSCs, which exhibited aberrant upregulation of Nrf2 in conjunction with an elevated transcriptional regulation of ABCG2, Bcl-2 and Bmi-1. Consequently, CSCs possess prolonged cell survival, infinite cell proliferation and highly resistant apoptosis. Following silencing of the function of Nrf2 the cervical CSCs became more sensitive to DNA targeting drugs and apoptosis. Our results suggested that Nrf2-mediated drug and apoptosis resistance are important in cancer therapies and tumor recurrence. Therefore, designing anticancer drugs targeting Nrf2 may be crucial to prevent CSC-mediated tumorigenesis.

Introduction

Cervical cancer is one of the most common gynecological malignancies, resulting in a significant mortality rate in women worldwide (1,2). In most cases, cervical cancer is treated by hysterectomy or primary radiotherapy after diagnosis at early stages (1,2). However, patients at an advanced stage of cancer progression (regional and distant metastases) are generally treated with the combination of radio and chemotherapy. Despite the significant improvements in cancer treatment strategies, the overall survival of cervical cancer patients has yet to be determined. After treatment, >75% of patients have recurrence within 1-2 years, resulting in tumor invasion and metastasis (3).

Studies conducted on different solid tumors reported that the persistence of cancer stem cells (CSCs) are the major factor responsible for therapy failure and tumor recurrence (4-7). A proposed cancer stem cell theory suggested that CSCs are a small subpopulation within the heterogenous tumor (7,8) and are highly potent with regard to differentiation, infinite proliferation rate, self-renewal, tumorigenesis and invasion (7-9). Therefore, it is important to isolate and characterize CSCs to gain a better understanding of CSC-meditated tumorigenesis and metastasis. CSCs have been isolated based on their property of Hoechst 33342 dye exclusion by using fluorescence-activated cell sorting (FACS) analysis. During the FACS analysis, these CSCs were reduced on the left side of the dot plot FACS analysis quadrant and designated as side population (SP) cells (10). These SP cells share all the significant features of CSCs and elevated ATP-binding cassette (ABC) transporter protein, while ABCG2 in SP cells has been shown to be involved in Hoechst 33342 efflux or multidrug resistance (11-13). In the present study, we aimed to characterize the cervical CSCs to gain a better understanding of Nrf2-mediated drug and apoptosis resistance of CSCs.

Materials and methods

Cancer samples and cell culture. Cervical cancer samples were collected from patients at the time of surgery in the Department of Obstetrics and Gynecology in accordance with the ethical principles approved by The Second Hospital of Jilin University, China. A total of 15 samples were collected by punch biopsies. The total number of patients was 30, with an age range of 39-43. Three poorly differentiated carcinomas (PDSCC), 15 high-grade SILs and 12 well-differentiated squamous cell carcinomas (WDSCC) were identified. The corresponding control non-malignant cervical epithelial tissues were obtained from healthy groups at the time of hysterectomy. The cancer tissues were then washed and further processed as previously described (14).

FACS analysis. By using a hemocytometer, ~10^6 cells were taken and divided into two groups. In group I, the cells were labeled with Hoechst 33342 dye alone (n=11) and in group II, the cells were treated with the reserpine drug and Hoechst 33342 dye (n=11). Subsequently, the cells were counterstained with propidium iodide (PI) 2 μg/ml and analyzed by flow cytometer.

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Assays. Cell resistance, Matrigel invasion, sphere formation and TUNEL assays were performed exactly as previously described (15,16).

Tumor cell implantation. The FACS sorted SP and non-SP cells (4x10³ cells) were administered into NOD/SCID mice by subcutaneously injection. The density of the injected cells and mice growth was monitored as previously described (17). After 3-4 weeks, the derived tumors from the sacrificed mice were removed.

Reverse transcription (RT)-quantitative polymerase chain reaction (qPCR). Total RNA was extracted and complementary DNA was prepared using Reverse Transcriptase kit (Fermentas). RT-qPCR analysis was performed on an iCycler IQ real-time detection system, using an IQ Supermix with SYBR-Green (both from Bio-Rad, Hercules, CA, USA). The sequences of human specific primers used were as follows (15,16): ABCG2 forward, TCA ATC AAA GTG CTT CTT TTT TATG and reverse, TTG TGG AAG AAT CAC GTG GC); Nrf2 forward, ACA CGG TCC ACA GCT CAT C and reverse, TGC CTC CAA AGT ATG TCA ATC A); GAPDH forward, ATG TCG TGG AGT CTA CTG GC and reverse, TGA CCT TGC CCA CAG CCT TG); Oct-4 forward, TCG AGA ACC GAG TGA GAG GC and reverse, CAC ACT CGG ACC ACA TCC TTC); Bmi-1 forward, CTCCCAACTGGTTCGACCTT and reverse, CGGTTTCCATATTTCTCAGT); CD133 forward, TCT TGA CCG ACT GAG AC and reverse, ACT TGA TGG ATG CAC CAA GCA C); EpCAM forward, CTG CCA AAT GTT TGG TGA TG and reverse, ACG CGT TGT GAT CTC CTT CT); BCL-2 forward, ACA CTG TTA AGC ATG TGC CG and reverse, CCA GCT CAT CTC ACC TCA CA); and Bax forward, GGA TGC GTC CAC CAA GAA and reverse, ACT CCC GCC ACA AAG ATG) (17-20). The PCR parameters used to set the PCR reactions were: Initial denaturation at -95°C for 15 sec; annealing at -58°C for 45 sec; and extension at -60°C for 30-45 sec, for 35 cycles. The amplified products were visualized by ethidium bromide-stained 1.2% agarose gels. Image J was used to measure the band intensity. The data presented in the graph are the average values of three independent experiments.

RNA interference. Small interfering RNA (siRNA) specific to the Nrf2 gene was purchased from Dharmacon (Lafayette, CO, USA). siRNA transfection (final concentration of 200 nm) was performed as per the Dharmacon protocol by using Oligofectamine 2000 (Invitrogen-Life Technologies, Carlsbad, CA, USA). The transfected siRNA cells were analyzed after 48 h.

Immunofluorescent staining. The SP cells and main population cells were seeded in cover slips on 12-well plates (1x10⁵ cells/well). The primary antibodies used were mouse anti-CD133 (1:100), anti-Oct-4 (1:200), anti-EpCAM (1:200), anti-ABCG2 (1:1,000), anti-bcl2 (1:100) and anti-Bax (1:200). After 24-h incubation in 1% BSA-TBS the antibodies were incubated overnight at 40°C. After washing with 1X PBS, the cells were incubated with secondary antibody conjugated with FITC (dilution: 1:1,000 in 1% BSA-TBS), at room temperature for 1-2 h. For immunohistochemistry, the tissues were processed with CD133 as previously described (21) and stained with mouse anti-CD133 (1:100) and rabbit anti-Nrf2. Tissues and cells were stained with Hoechst 33342 (dilution: 1:100; Bio-Rad) dye to visualize the nucleus.

The immunostaining of squamous spheres were performed as previously described (12). Spheres were fixed onto glass slides in ice-cold 4% paraformaldehyde (40°C for 10 min), blocked with normal serum for 30 min, and incubated with mouse monoclonal anti-Oct-4 and CD44 (1:200; Chemicon, Japan) overnight. After being washed with PBS, the slides were incubated with FITC-conjugated chicken anti-rat IgG overnight in the dark. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The cells and tissues were subsequently viewed under a confocal laser scanning microscope (Leica TCS, Mannheim, Germany). Image analysis and figures were prepared using Adobe Photoshop CS6.

Western blot analysis. Proteins were extracted from the SP and non-SP cells, and protein concentration was determined using the Bradford assay (15). Primary antibodies (rabbit anti-human ABCG2, Nrf2 and GAPDH) were incubated overnight, followed by a secondary antibody (goat anti-rabbit IgG with alkaline phosphatase markers) and a chemiluminescence reagent. Blots were detected using gel documentation and scanned using a densitometer (GS-710; Bio-Rad).

Statistical analysis. A one-way analysis of variance (ANOVA) and Student's t-test were performed to determine the significant difference between the treatment and control groups. A probability level of $P<0.05$ or 0.01 was considered statistically significant.

Results

Flow cytometry-based purification of cancer stem-like SP cells. In the FACS analysis, cells passing through the laser beam were considered forward and side scatter cells correlating with cell size and population density, respectively. Using PI staining, dead cells were excluded and the main population was gated as a P1 region. The cancer cells that efficiently effluxed the DNA binding dye Hoechst 33342 and were reduced in the left lower quadrant of the FACS profile were termed as 'side population (SP)' cells. We found ~3.1% of SP cells (P2-gated population) from cervical cancer samples, which actively pumped out the Hoechst 33342 dye (Fig. 1A). The process of drug expulsion by SP cells occurred due to the overexpression of the ABC transporter protein, ABCG2. As a confirmatory test we treated the cervical cancer samples with the ABC inhibitor reserpine and analyzed using FACS. Following treatment with reserpine, the SP cell population was dramatically reduced to 0.9% (Fig. 1B). Thus, the results suggested that the presence of ABC proteins in SP cells is significant for the expulsion of DNA binding dyes.

Cervical cancer SP cells possess properties of embryonic stem cells. The FACS sorted SP and non-SP cells were further analyzed for the association of stem cell features. First, we evaluated the expression of stem cell surface genes, such as Oct-4, CD133 and EpCAM, between the SP and non-SP cells using RT-PCR analysis. As shown in the graph (Fig. 2A), the transcriptional regulation of the abovementioned genes are
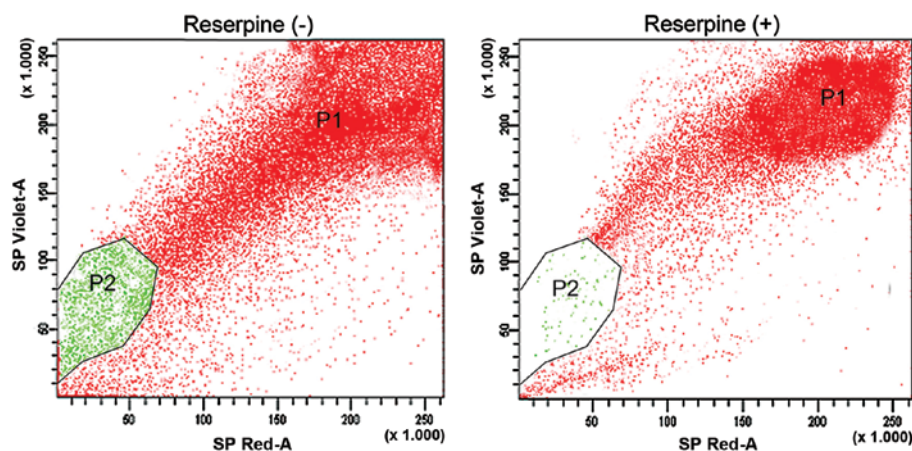
Figure 1. Purification of CSCs by FACS analysis. (A) Presence of SP cells of ~3.1% from cervical cancer samples (P2 gated region). P1 indicates the main live cell population. (B) The percentage of the SP cells was significantly reduced to 0.7% following treatment with reserpine, an inhibitor of the ABC transporters. CSCs, cervical cancer stem cells; SP, side population; ABC, ATP-binding cassette; FACS, fluorescence-activated cell sorting.
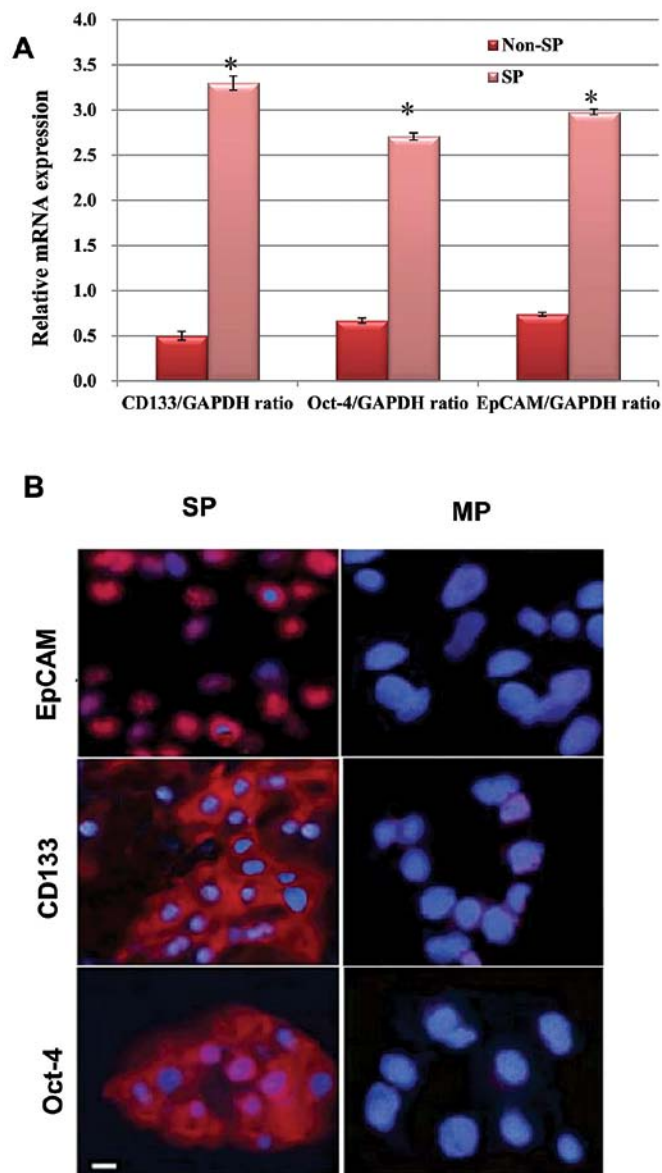

Figure 2. Enhanced stemness gene expression profile in FACS sorted SP cells. (A) Quantification graph generated from RT-PCR analysis showing upregulated mRNA transcriptional regulation stemness genes, such as CD133, EpCAM and Oct-4 in SP cells. (B) Immunocytochemistry of FACS sorted SP cells showed more positivity towards the stem cell surface proteins, whereas the non-SP cells showed either null or very weak positivity. The bar represents the standard deviation. *P<0.01. Scale bar, 10 μm. SP, side population, FACS, fluorescence-activated cell sorting.
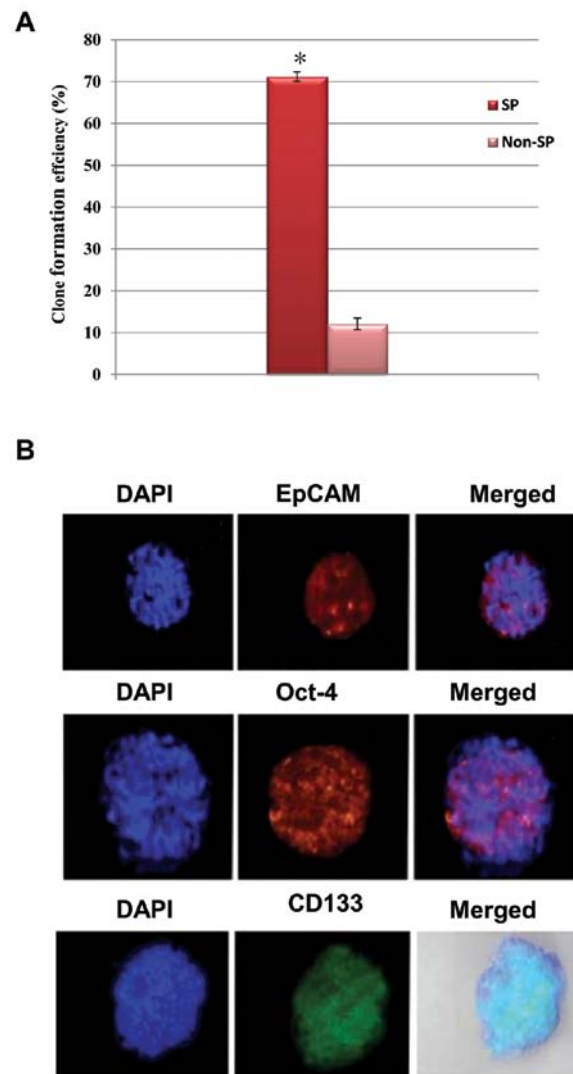
Figure 3. Cervical cancer SP cells are highly potent in generating tumor spheres. (A) Clone formation efficiency graph showing that the SP cells are rapidly generating more tumor spheres than non-SP cells. (B) Immunofluorescence staining of SP cell-induced tumor spheres exhibit positivity towards CD133, EpCAM and Oct-4. The error bar shows the standard deviation. *P<0.01. SP, side population.

significantly upregulated in the SP cells than non-SP cells. In addition, our immunofluorescence assay revealed that the SP cells exhibited increased positivity towards these proteins, whereas the non-SP cells exhibited null or less positivity towards these proteins (Fig. 2B). Thus, the over-expression of these stem cell proteins may be involved in the self-renewal process. Therefore, we performed a sphere formation assay to determine the ability of self-renewal of SP cells. As expected, the SP cells produced significantly more tumor spheres and generally the tumor size was much larger than that of the SP cells (Fig. 3A). Of note, the spheres generated by the SP cells were highly positive towards stem cell surface proteins such as CD133, EpCAM and Oct-4 (Fig. 3B). Therefore, an increased expression of stemness genes in SP is crucial for the maintenance of self-renewal of the cancer stem-like SP cells.

Elevated Nrf2 attenuates apoptosis and multidrug intake. We performed a drug resistance assay to compare the cell survival rate between the SP and non-SP cells. As shown in Fig. 4A, the SP cells were more resistant to 5-fluorouracil (5-FU),

cisplatin, paclitaxel and oxaliplatin. The survival rates of the SP cells were significantly higher (>75%) following treatment with the multidrugs than the non-SP cells (<20%). It has been shown that the aberrant upregulation of Nrf2 was involved in the regulation of ABC genes, thus ultimately resulting in chemotherapy resistance of the SP cells (22). Therefore, we evaluated the transcriptional expression of the Nrf2 gene in the SP and non-SP cells. The relative mRNA expression of the Nrf2 gene was significantly enhanced in the SP cells in conjunction with the elevated mRNA expression of the ABC transporter ABCG2 and anti-apoptotic genes such as Bcl-2 and Bmi-1 than in the non-SP cells (Fig. 4B). However, Bax gene expression was significantly reduced in the SP cells as compared to the non-SP cells. In addition, our immuno-fluorescence results revealed that the cervical cancer SP cells exhibited enhanced positivity towards ABCG2 and Bcl-2, but decreased positivity towards Bax proteins as compared to the non-SP cells (Fig. 5A). As a consequence of the aberrant regulation of the ABCG2 and anti-apoptotic gene expression profiles, the number of SP cells that underwent apoptosis was markedly decreased compared to that of the non-SP
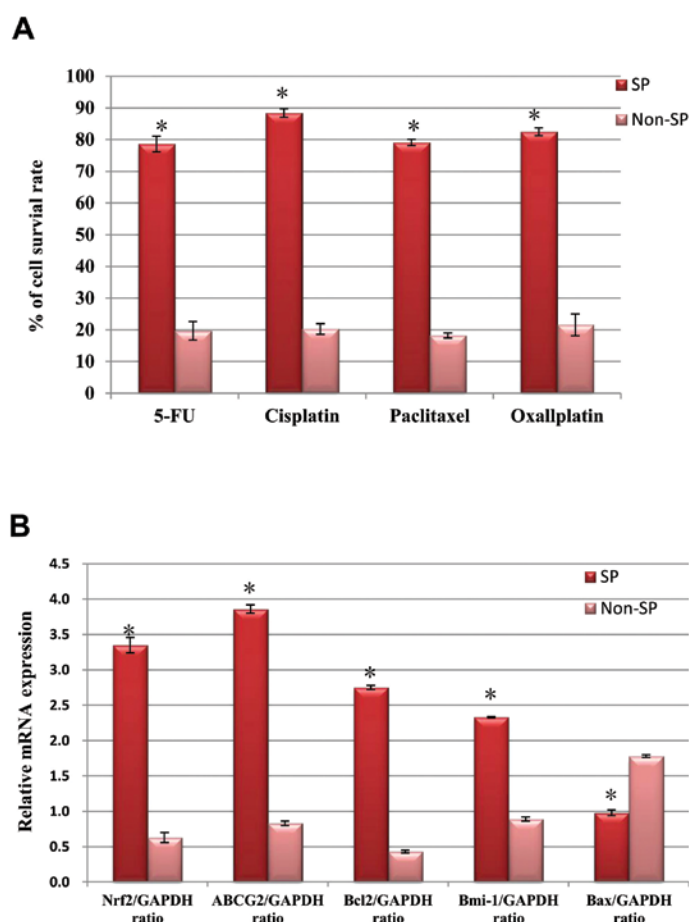
Figure 4. Elevated Nrf2 mediates enhanced cell survival of SP cells. (A) SP cells are highly resistant to 5-FU, cisplatin, paclitaxel and oxaliplatin. The SP cells have a higher survival rate (>75%) following treatment with these drugs compared to the non-SP cells (<20%). (B) Quantification graph generated from RT-PCR analysis showing that the relative mRNA expression profile of Nrf2 in SP cells was highly elevated in conjunction with ABCG2, Bcl-2 and Bmi-1 than in non-SP cells. Bar, standard deviation. *P<0.01. 5-FU, 5-fluorouracil; SP, side population.
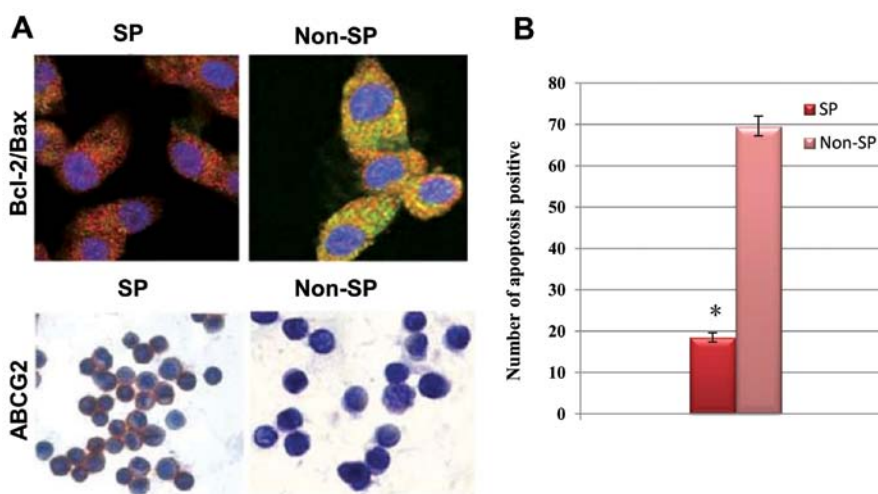

Figure 5. Elevated Nrf2 reduces programmed cell death rate in SP cells. (A) Immunofluorescence staining of SP cells showing an enhanced expression of anti-apoptotic factor Bcl-2, Bax and ABCG2 than in the non-SP cells. (B) The number of SP cells positive for apoptosis are significantly less than in the non-SP cells. Bar, standard deviation. *P<0.01. SP, side population.

cells (Fig. 5B). These results suggested that the elevated expression of Nrf2 is crucial in the long-term survival of cancer stem cells by upregulating and downregulating the drug efflux and apoptosis mechanism, respectively.

siRNA interference of Nrf2 enhances drug and apoptosis sensitivity of SP cells. We examined whether the rapid inactivation of Nrf2 downregulated the ABC transporter and anti-apoptotic genes. Thus, we used a silencing RNA approach to inactivate
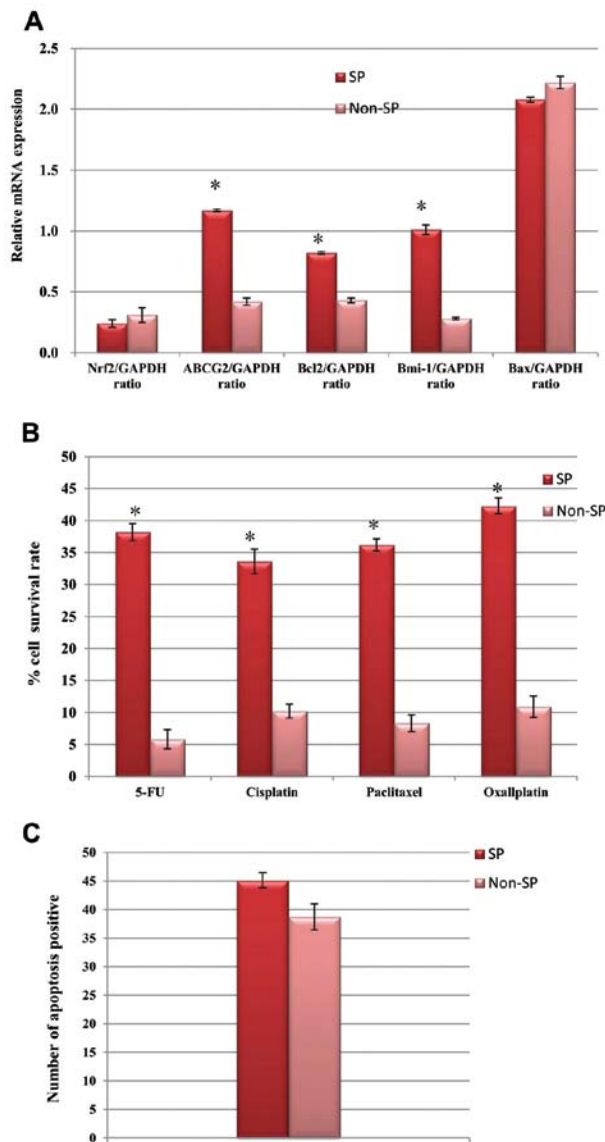
Figure 6. siRNA interference of Nrf2 enhances the drug and apoptosis sensitivity of SP cells. (A) After rapid inactivation of Nrf2, the transcriptional regulation of ABCG2, Bcl-2, Bmi-1 and Bax are significantly downregulated and upregulated, respectively. (B) Comparison of cell survival rate of SP and non-SP cells after Nrf2 knockdown. (C) Evaluation of the apoptotic rate in SP and non-SP cells in Nrf2-silenced SP and non-SP cells. Bar, standard deviation. *$P<0.05$; **$P<0.01$. SP, side population.

the Nrf2 gene rapidly and temporarily. After silencing the Nrf2 gene in the SP cells, the transcriptional regulation of the ABCG2, Bcl-2 and Bmi-1 genes was significantly downregulated (Fig. 6A). Consequently, the SP cells were more sensitive towards multidrug treatment, as the survival rate of SP cells was significantly reduced (Fig. 6B). Similarly, the number of SP cells that underwent apoptosis was significantly elevated after Nrf2 inactivation (Fig. 6C). These results suggested that an elevated Nrf2 is a major causative factor for chemotherapy failure and poor survival rate of cancer patients.

Nrf2-upregulated SP cells are highly tumorigenic and invasive. To determine the tumorigenicity of FACS-sorted Nrf2-overexpressed SP cells, we implanted the lowest density ($4x10^3$ cells) of the SP and non-SP cells separately into the NOD/SCID mice. The SP cells were highly potent for initiating rapid tumor growth in the NOD/SCID mice and the tumor size was larger (Fig. 7A), whereas the non-SP

cells failed to generate tumors at this cell concentration. The Matrigel invasive assay revealed that the SP cells were highly invasive (Fig. 7B). Furthermore, the tumor tissues derived from SP-induced cells showed an enhanced Nrf2 and Bcl-2 staining compared to the non-SP tissues (Fig. 8). These results clearly suggested that the presence of SP cells are the major factor involved in tumor recurrence, invasion and metastasis after chemotherapy failure.

Discussion

The heterogenous population of cancer cells contains a small proportion of tumor-initiating cells termed cancer stem cells (CSCs), responsible for multidrug therapy failure and tumor relapse as they are capable of self-renewal (23-25). CSCs have been isolated and characterized in different solid tumors such as pancreatic carcinoma, ovarian and prostate cancer (4-7). Additionally, CSCs are highly aggressive and resistant
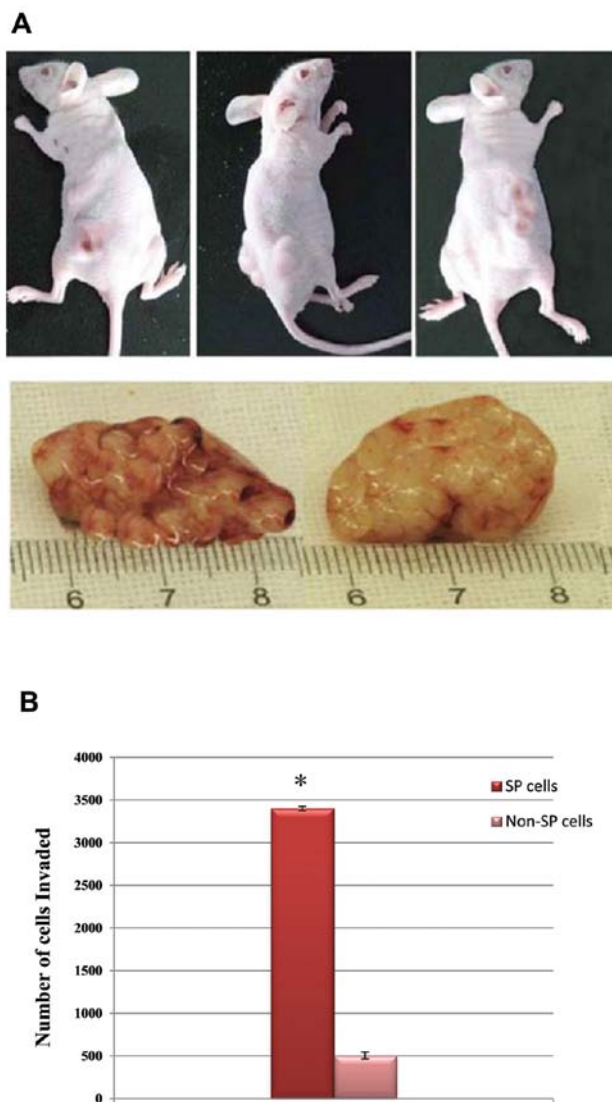
Figure 7. Nrf2 overexpression SP cells are highly tumorigenic and invasive. (A) The tumor sections derived from SP and non-SP cells after 23 days of subcutaneous injection into NOD/SCID mice. (B) Matrigel invasion assay showing the number of SP cells invading the matrigel are significantly higher than the non-SP cells. Bar, standard deviation. *P<0.01. SP, side population.
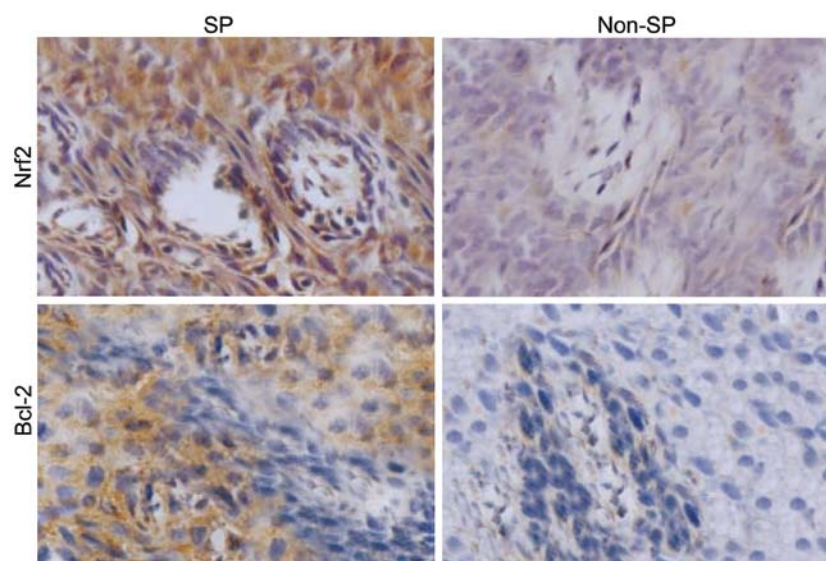

Figure 8. Immunohistochemical staining of SP and non-SP derived tumor tissues. SP cell-derived tumor tissues show enhanced positivity towards Nrf2 and Bcl-2. SP, side population.

to multidrug treatment and apoptosis (4-7). The increased multidrug resistance properties of CSCs are mainly due to the higher expression levels of drug efflux pumps such as ABC transporters ABCG2 and Bcrp1 which are encoded by multi-drug-resistant (MDR) gene 1 (12). However, investigations into CSCs to identify novel signaling and aberrantly upregulated proteins in CSCs may be useful to developing novel and effective anticancer drugs.

In the present study, by using the Hoechst 33342 dye exclusion assay we have isolated and characterized the cancer stem-like SP cells from cervical cancer samples. We have found a significant amount (3.1%) of cervical cancer SP cells. The SP cell population possesses overexpression of ABC transporter proteins such as ABCG2. Similarly, it has been previously showed in cervical cancer that HeLa stem cells possess the highly expressed MDR protein Bcrp1 (3). Furthermore, we have demonstrated that FACs-sorted cervical cancer SP cells are capable of self-renewal as they express higher levels of stem cell surface proteins such as CD133, CD44, EpCAM and Oct-4, which are involved in the maintenance of self-renewal. These proteins are also responsible for the higher cell proliferation rate and rapid generation of tumor spheres because the FACs-purified SP cells are able to initiate tumor spheres. These tumor spheres are highly positive for CD133, CD44 and Oct-4. With regard to these findings, it has been previously shown that Bcrp1^{+} cells underwent rapid proliferation and they are capable of self-renewal (3,26). Another interesting phenotype of the SP cells we identified is that they are highly resistant to multidrugs and apoptosis. Furthermore, these SP cells can initiate tumor growth in NOD/SCID mice even at the lowest cell concentration, confirming that SP cells are capable of causing tumor recurrence and invasion following therapy failure. Similarly, the Bcrp1^{+}/CD44^{+} cervical cancer SP cells are highly tumorigenic and induce rapid tumor growth in NOD/SCID mice (9). Overexpression of the stem cell surface protein CD133 in different types of cancer has been shown to be involved chemoresistance, self-renewal and tumorigenesis *in vitro* and *in vivo* (27). However, the signaling mechanism and downstream pathways involved in drug resistance, apoptosis resistance and tumor recurrence remain to be determined.

The major role of Nrf2 is providing protection to the cells from oxidative stress and other environmental cues, such as xenobiotics. Generally, the downstream signaling of Nrf2 leads to activation of several antioxidant enzymes and an elevated expression of various drug efflux transporter proteins on the cell surface (28-30). On the other hand, negative regulation of Nrf2 leads to ubiquitin-mediated proteosomal degradation by KEAP1 (31,32). Loss of Nrf2-KEAP1 interaction or point mutations in KEAP1 has been found to lead to the upregulation of the Nrf2 gene, which is often present in primary tumors (33,34). Studies in lung cancer stem cells reported that upregulated Nrf2 leads to the overexpression of ABC transporter proteins, contributing to chemotherapy failure (2,12,17). Furthermore, depletion of Nrf2 leads to the suppression of ABC transporter gene expression and its function (22). Similarly, we have demonstrated that cervical cancer SP cells possess an aberrantly elevated level of the Nrf2 transcript and consequently the transcriptional regulation of ABCG2 and Bcl2/Bmi-1 are highly upregulated. Furthermore, we have shown that silencing of the Nrf2 gene

expression attenuated the expression of ABCG2, Bcl-2 and Bmi-1, rendering the SP cells more sensitive to drug treatment and efficiently subjected to apoptosis. There is a high risk of alteration in programmed cell death during carcinogenesis associated with mutation in the p53 gene and modulation of apoptotic regulatory Bcl-2, Bmi-1 and Bax proteins in cervical CSCs (18). Inactivation of the p53 gene induces the production of other pro-apoptotic proteins such as Bax and represses the anti-apoptotic factors such as Bcl-2 and Bmi-1 (18). Therefore, we hypothesized that an elevated level of Nrf2 may be involved in the inactivation of the p53 gene and its function.

Taken together, our results suggest that elevated Nrf2 signaling in cervical cancer SP cells are a major root cause for DNA targeting drug and cell death resistance. Future studies concerning elucidation of the precise molecular mechanism behind the regulatory pathways and additional factors involved in Nrf2-mediated ABCG2/Bcl-2 overexpression may provide insight into the development of novel anticancer drugs that effectively suppress Nrf2 signaling and the function of drug efflux pumps.

Acknowledgements

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