Identification of Cancer Stem Cell–Like Side Population Cells in Human Nasopharyngeal Carcinoma Cell Line

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Abstract

Side population (SP) cells have been isolated from several solid tumors. They lack distinct molecular markers for cancer stem cells (CSC) and increasing evidence suggests that they may play an important role in tumorigenesis and cancer therapy. However, there are no reports about the existence and function of SP cells in nasopharyngeal carcinoma (NPC) cells thus far. In this study, we scanned SP cells from five NPC cell lines and investigated stem cell characteristics, such as proliferation, self-renewal, and differentiation, using SP cells from the widely-used CNE-2 NPC cell line. We observed a strong tumorigenesis ability of SP cells following *in vivo* transplantation into nonobese diabetic/severe combined immunodeficient mice. Immunofluorescence revealed that cytokine 19 was highly expressed on SP cells. SP cells were found to be more resistant to chemotherapy and radiotherapy and this was related to the ATP-binding cassette half transporter member 2 of G family protein and Smoothened protein expression, respectively. Our results not only showed that SP cells in human NPC cell line CNE-2 had stem cell characteristics *in vitro* but also showed that they had a strong ability to form tumors *in vivo*. Importantly, we found the cell marker, cytokine 19, may serve as a potential molecular marker for further characterization of CSC. Taken together, our data shed light on tumorigenesis and therapeutic-resistant mechanisms, which are helpful for developing novel targets for effective clinical treatment of NPC. [Cancer Res 2007;67(8):3716–24]

Introduction

The concept of cancer stem cell (CSC) was introduced many years ago to explain tumor cell heterogeneity, and recent studies suggest that CSC may be responsible for tumorigenesis and contribute to some individuals' resistance to cancer therapy. Recently, CSC have been isolated from several human tumors that have markers for putative normal stem cells, including leukemia, breast cancer (1), brain tumors (2), prostate cancer (3), and ovarian cancer (4). CSCs are more important than other tumor cells because they are capable of self-renewing, differentiating, and maintaining tumor growth and heterogeneity, playing an important role in both tumorigenesis and therapeutics. However, research has been hampered by the lack of distinct molecular makers on CSC. Fortunately, analysis of the hematopoietic system has shown that bone marrow stem cells contain a subpopulation that effluxes the DNA binding dye, Hoechst 33342, out of the cell membrane. These cells are called side population (SP) cells and are shown to have stem cell characteristics and enrich the stem cell population (5–7).

Hoechst 33342 is a cell-permeable DNA-specific bisbenzimidazole dye. Its ability to efflux out of cells is based on the ATP-binding cassette (ABC) half transporter member 2 of G family protein (ABCG2; also termed mitoxantrone resistance gene), which is encoded by 655 amino acids on chromosome 4q22, and confers multidrug resistance to mitoxantrone and topotecan (8). It is now possible to obtain CSC-like SP cells using a fluorescence-activated cell sorting (FACS) techniques based on Hoechst 33342 efflux. SP cells have already been found in many normal tissues and several solid tumors, including glioblastoma (9), gastrointestinal system tumor (10), head and neck squamous carcinoma (11), hepatocellular cell lines (12), and even primary cultures from neuroblastoma patients (13).

Although research is rapidly advancing in this field, to our knowledge, there are no published reports examining the role of SP cells in human nasopharyngeal carcinoma (NPC). NPCs are fairly rare among Caucasians in Western Europe and North America, but there is a higher incidence in Southern China, especially in people of Cantonese ancestry in the Guangzhou region (14–16). The disease is associated with multiple factors, including living condition, genetics, viral infection, and environment. Although advances in radiation and chemotherapy have improved the prognosis of individuals with NPC, many patients still suffer from the disease as a result of therapeutic resistance and regional lymph node metastasis. Thus, it is worth studying CSC to better understand its origin and progression. Because surgery is not recommended for NPC patients, it is often difficult to obtain clinical tissue for primary culture. Consequently, we investigated the prevalence of SP cells and explored CSC using a human NPC cell line. Improving our understanding of CSC may open up new fields of basic and clinical tumor research. Strikingly, we identified CSC-like SP cells in human NPC cell line that may aid the development of novel therapeutic strategies and efficient drugs that target NPC CSC.

Materials and Methods

Cell culture. A well-differentiated human NPC cell line, CNE-1, and four poorly differentiated cell lines, CNE-2, SUNE-1, HONE-1, and C-666-1 (conserved in our laboratory), were maintained in RPMI 1640 culture (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 μg/mL streptomycin. After sorting, some cells were cultured in UltraMEM culture (Cambrex, North Brunswick, NJ) supplemented with 5% FBS, 10 ng/mL basic fibroblast

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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growth factor (bFGF; Sigma-Aldrich, St. Louis, MO), 10 ng/mL epidermal growth factor (EGF; Sigma-Aldrich), 1 mmol/mL L-glutamine (Sigma-Aldrich), 50 units/mL penicillin G, and 50 μg/mL streptomycin. All cells were maintained in a humidified 5% CO_2 incubator at 37°C.

Sorting NPC cell lines by FACS. Once the cells had reached a logarithmic growth phase, they were analyzed by FACS. Cells were digested with 0.25% trypsin (Sigma-Aldrich), washed twice with calcium/magnesium–free PBS, resuspended in ice-cold RPMI 1640 culture (supplemented with 2% FBS) at a concentration of 1×10^6 cells/mL, and incubated at 37°C in a 5% CO_2 incubator for 10 min. The DNA binding dye, Hoechst 33342 (Sigma-Aldrich), was then added at a final concentration of 5 μg/mL and incubated for 90 min in the dark with interval mixing. The cells were washed twice with PBS, 1 μg/mL propidium iodide (Sigma-Aldrich) was added, and the cells were kept at 4°C in the dark before FACS (FACSDiva Option, Becton Dickinson, Mountain View, CA) sorting using dual-wavelength analysis. Because Hoechst 33342 extrudes from cells using verapamil (a calcium ion tunnel antagonist)–sensitive ABC transporters, a subset of the cells were incubated with 50 μmol/L verapamil for 30 min at 37°C before adding Hoechst 33342 to determine whether this would block the fluorescent efflux of SP cells in CNE-2. We collected both SP and non-SP (NSP) cells to evaluate sorting purity and conduct further experiments.

Cell growth rate and cell cycle analysis. Freshly sorted SP and NSP cells were incubated at 500 cells per well in 96-well plate triplicates and cultured in complete RPMI 1640 and UltraMEM to observe the growth rate. During the 8 days, we took photos of the cells, measured the absorbance of the cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (17), and drew the cell growth curve according to the data.

After some of the sorted cells were cultured for several days (<1 week), we harvested 1×10^6 cells for cell cycle analysis. The cell suspension was washed twice with PBS and fixed dropwise with 2 mL of 70% ice-cold ethanol for 18+ h at 4°C. The cells were washed twice with PBS, RNase was added at a final concentration of 20 μg/mL to avoid staining the RNA, and the cells were incubated at 37°C for 30 min. The cells were washed once with PBS, propidium iodide was added at a final concentration of 15 μmol/L, and after 5 min, they were analyzed by flow cytometry. The proliferation index (PI) was calculated using the formula PI = $(G_2M + S) / (G_0G_1 + S + G_2M)$ to reflect the percentage of cells in the proliferation phase. The S-phase cell fraction (SPF) reflected the cell percentage in the S phase and was calculated using the formula SPF = $S / (G_0G_1 + S + G_2M)$.

Clone formation and long-term differentiation of SP and NSP cells. Freshly sorted SP and NSP cells were counted, plated in triplicate at 200 cells per well in six-well plates, and cultured with RPMI 1640 complete culture for ~10 days. After most cell clones had expanded to >50 cells, they were washed twice with PBS, fixed in methanol for 15 min, and dyed with crystal violet for 15 min at room temperature. After washing out the dye, we counted the clone number that contained >50 cells and compared the results. The clone formation efficiency (CFE) was the ratio of the clone number to the planted cell number.

The differentiation assay was done 18 days after cell sorting. Cells were cultured in normal RPMI 1640 to reach the desired cell number. Then, the cultured SP and NSP cells were stained with Hoechst 33342 and analyzed by FACS to quantitate the proportion of SP cells and determine the differentiation ability of the two subpopulations.

Transcription of ABCG2 in SP and NSP cells. Total cell RNA was extracted from newly sorted SP and NSP cells using Trizol reagent (Invitrogen, San Diego, CA) and reverse transcriptions were done according to the manufacturer's instructions (Invitrogen). The ABCG2 cDNA primers for PCR were 5′-GGGTTCTCTTCTTCCTGACGACC-3′ (forward) and 5′-TGGTTGTGAGATTGACCAACAGACC-3′ (reverse) and the final product was 398 bp long. The internal reference gene was *GAPDH*, primer sequences were 5′-GAGTCAACGGATTTGGTCGT-3′ (forward) and 5′-GACAAGCTTCCCGTTCTCAG-3′ (reverse), and the product was 185 bp long. Thermal cycle conditions included an initial incubation at 94°C for 4 min followed by 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 45 s and maintenance at 72°C for 7 min. The products were analyzed by electrophoresis on 1.5% agarose. We also analyzed expression of the ABCG2 proteins on the SP and NSP cell surface using flow cytometry 18 days after sorting with the monoclonal antibody (antihuman ABCG2, clone 5D3, conjugated with phycoerythrin; eBioscience, San Diego, CA) or its isotype control (phycoerythrin mouse IgG2b; eBioscience).

Tumor formation in an animal model. Nonobese diabetic (NOD)/severe combined immunodeficient mice (SCID) mice were purchased from the animal institute of the Chinese Academy of Medical Science and Peking Union Medical College (CAMS and PUMC) and maintained in microisolator cages. All experiments were approved by the animal care committee of CAMS and PUMC. Freshly sorted SP and NSP cells suspended in 200 μL PBS were inoculated into the axillary fossa of 6- to 7-week-old NOD/SCID female mice on the afternoon of the sorting day. The mice were monitored twice weekly for palpable tumor formation and euthanized 4 or 9 weeks after transplantation to assess tumor formation. Tumors were measured using a Vernier caliper, weighed, and photographed. A portion of the s.c. tumor tissue was collected, fixed in 10% formaldehyde, and embedded in paraffin for H&E staining to assess tumor pathology.

Immunofluorescence analysis of the expression of cytokines 8, 18, and 19 on SP and NSP cells. Cytokines are indispensable markers for classification of poorly differentiated or phenotypically "deviant" tumors. Cytokines 8, 18, and 19 are prevalent in squamous cell carcinoma, and specific antibodies against them aid in diagnosing NPC. To determine whether there were expression differences in the three cytokines between SP and NSP cells, freshly sorted cells were grown on sterile glass cover slides overnight at 37°C, washed twice with PBS, and fixed in 4% paraformaldehyde for 30 min at 4°C. The cells were washed twice more with PBS, and incubated with 5% bovine serum albumin for 2 h at room temperature to block nonspecific binding of IgG. After two further PBS washes, the cells were incubated overnight at 4°C with primary antibodies against cytokines 8, 18, and 19 (Santa Cruz Biotechnology, Santa Cruz, CA) that had been diluted in PBS according to the manufacturer's instructions. We then washed the slides twice with PBS containing 0.02% Tween 20 (PBS-T) and added fluorochrome-conjugated secondary antibody at room temperature for 2 h in a dark chamber. The cells were washed thrice with PBS-T and coverslipped with aqueous mounting medium (containing 0.5 mg/mL 4′,6-diamidino-2-phenylindole to stain the nuclei). We took random photos (Olympus BX51, Tokyo, Japan) of eight visual fields using 100-fold magnification and counted the total cell and positive cell numbers to calculate the expression percentage.

Radiation and drug sensitivity assay. We exposed SP and NSP cells to X-ray to determine their radiation sensitivity differences. We seeded 200 freshly sorted SP and NSP cells per well of a 12-well plate, each in six wells, three of which were irradiated with 2 Gy of X-ray (400 cGy/min, using a 12 × 6 cm irradiation field) the day after seeding. The other three wells were not exposed to X-ray and cultured as controls under normal conditions. When most cell clones had reached >50 cells, we stained with crystal violet to determine the clone number that would reflect the ability of cells to survive irradiation. According to Shafaee et al. (18), cyclopamine, which is a naturally occurring steroidal alkaloid that specifically blocks the sonic hedgehog (SHH) signaling pathway by interacting with the protein, SMO (19), may improve the radiation sensitivity of pancreatic cancer cells. To determine whether cyclopamine could improve the radiation effect on NPC, we planted 400 newly sorted cells per well into a six-well plate and incubated them with medium containing 2 μmol/L cyclopamine (Biomol International, Plymouth Meeting, PA). After 36 h, we replaced the medium with PBS, exposed the cells to 2 Gy X-rays, and cultured them under normal conditions for a week before staining as described above. To determine whether the phenomenon was involved with protein SMO, we conducted a Western blot to detect SMO levels in the SP and NSP cells (the first two passages of newly sorted) using an anti-SMO monoclonal antibody (Santa Cruz Biotechnology) according to methods described by Bijlsma et al. (20). We loaded 30 μg total cell protein from each specimen.

For the drug sensitivity assay, freshly sorted SP and NSP cells were planted at 700 cells per well in 96-well plates. Mitoxantrone was added the following day in a concentration gradient and repeated in four wells. The other four parallel wells were preincubated with 50 μmol/mL verapamil as a chemosensitizer to mitoxantrone for 30 min. The culture medium was

then replaced with different concentrations of mitoxantrone. After three days, the cell survival rate (SR) was determined using the MTT method. SR was calculated using the formula SR = (mean absorbance of the test well / mean absorbance of the control) × 100%; inhibition rate (IR) was calculated using the formula (IR) = 100% − SR. We also exposed freshly sorted SP and NSP cells to cisplatin (an antitumor drug used to treat NPC) and mitomycin C (a non-NPC sensitive drug) to determine differences in drug sensitivity using the MTT method.

Statistical methods. Microsoft Office Excel 2003 and the statistical software SPSS12.0 were used in data processing and in analyzing the significance between SP and NSP cells with the unpaired or paired *t* test. *P* values <0.05 were considered significant. Data were expressed as the mean ± SD from at least three independent experiments.

Results

Cell sorting. After excluding dead cells and cellular debris based on scatter signals and propidium iodide fluorescence, the NPC cell line, CNE-2, was sorted. The P3 gate showed the SP cells that were Hoechst 33342 negative/dim, and the P4 gate indicated the NSP cells that were Hoechst 33342 positive (Fig. 1*A*). SP cells occupied ~2.6% of the total cells. When preincubated with verapamil for 30 min, the percentage of SP cells dropped to 0.2% of the total cells (Fig. 1*B*), which is consistent with reports that Hoechst 33342 exclusion is verapamil sensitive. SP (P3) and NSP (P4) cells were collected for subsequent experiments; the purity of SP cells was 99% and the purity of NSP cells was 96% (Fig. 1*C* and *D*). The other four human NPC cell lines, C-666-1, SUNE-1, HONE-1, and CNE-1, also contained a small subpopulation of SP cells and their proportions were 0.1%, 6.8%, 1.8%, and 0.7%, respectively (Supplementary Fig. S1).

Cell growth rate. MTT methods were conducted to determine the cell growth rate on the 2nd day after sorting. After day 4, the SP cells had reached a logarithmic growth phase in RPMI 1640, and by day 8, they had reached a plateau. In contrast, NSP cells continued growing slowly in RPMI 1640 until day 8, before moving into an upgrade phase, which was much slower than the SP cells ($P < 0.001$; Fig. 2*A*). When maintained in UltraMEM, SP cells also grew faster than NSP cells ($P < 0.001$). The growth of SP cells was no different between RPMI 1640 and UltraMEM, as was also true for NSP cells. However, cell morphology changed remarkably in UltraMEM (Fig. 2*B*). SP cells lost their normal appearance in UltraMEM culture and began to extend fibroblast-like filaments. NSP cells also showed morphologic changes, although these were not as striking as the SP cells. Cell cycle analysis of cells cultured in normal RPMI 1640 revealed no statistical differences in PI or the SPF ($P = 0.054$ and 0.197, respectively), which was in accord with the results of Al-Hajj et al. (1). Thus, strong tumorigenicity was not the result of differences in cell cycle distribution.

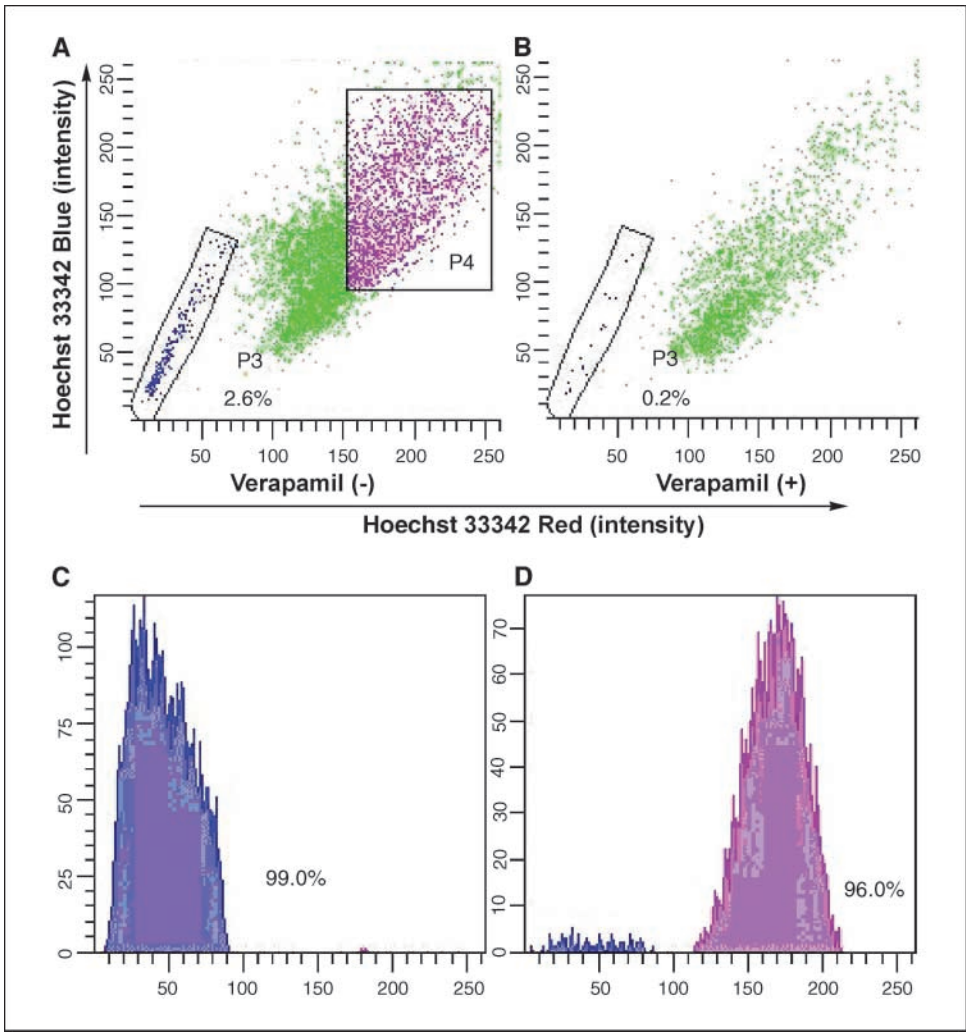

Figure 1. Cell sorting results and sorting purity. *A,* sorting of CNE-2 cells using Hoechst 33342. The P3 gate represents the SP cells (2.6% of total cells) and the P4 gate represents the NSP cells that were collected for subsequent research. *B,* the SP proportion was reduced to 0.2% when the cells were preincubated with verapamil to block the ATP transporter. *C* and *D,* the sorting purity of the freshly sorted SP and NSP cells was 99.0% and 96.0%, respectively.

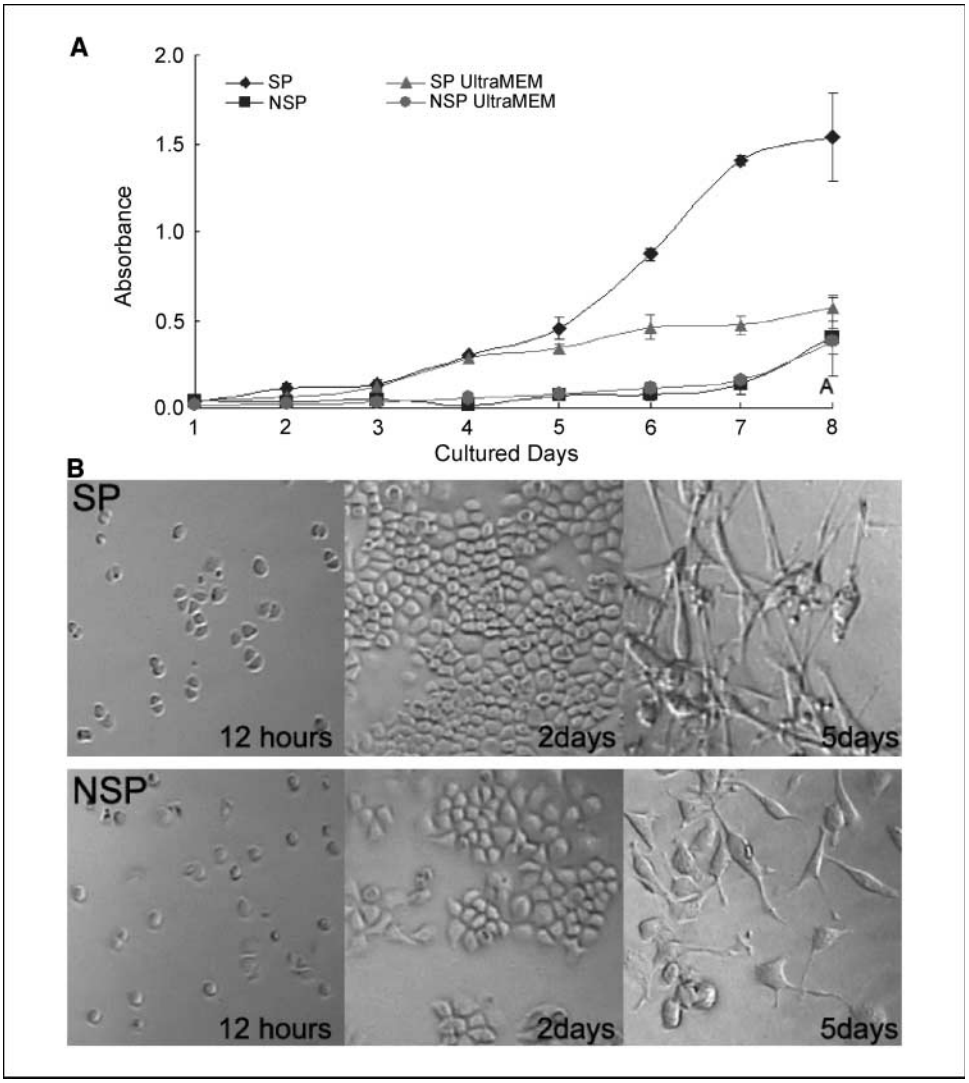

Figure 2. Cell growth curve and morphologic changes of SP and NSP cells in different culture media. *A,* the growth curves of SP and NSP cells cultured in normal RPMI 1640 and UltraMEM for 8 d. SP cells grew faster than NSP cells in both media ($P < 0.001$). Although SP cells grew more slowly in UltraMEM than in RPMI 1640, this difference was not significant. *B,* cell morphology changes of freshly sorted SP and NSP cells after seeding for 12 h, 2 d, and 5 d in UltraMEM. As the time increased, cells grew many long filaments, causing them to resemble fibroblasts but not polygon squamous epithelial cells. The changes in shape were more obvious in SP cells than in NSP cells.

Clone formation and long-term differentiation ability of SP and NSP cells. Clone formation assays were repeated twice in triplicate. After 9 days of culture, most clones had reached >50 cells. We counted the clone number and found that the mean CFE was 41.0 ± 1.0% and 9.5 ± 1.3% in SP and NSP cells, respectively. Statistical analysis showed significant differences in CFE between them ($P < 0.001$; Fig. 3*A* and *B*).

After culturing for ~20 days, we stained SP and NSP cells with Hoechst 33342 to reanalyze the SP proportion and measure differences in the differentiation ability of the two cell types. The data showed that SP cells still contain 9.6% Hoechst 33342 dull cells, whereas NSP cells only contain 0.4% (Fig. 3*C* and *D*), and these may have arisen from residual SP cells from the last sorting. This indicated that SP cells can differentiate into NSP cells, but NSP cannot differentiate into SP cells. These findings show that SP cells may undergo asymmetrical division to self-renew and generate heterogeneous phenotypes of low-tumorigenic cells, like NSP cells that form the bulk of the tumor. In contrast, NSP cannot differentiate into SP cells under normal circumstances.

***ABCG2* gene expression in SP and NSP cells.** Total cell RNA was extracted from SP and NSP cells and reverse transcription-PCR (RT-PCR) was done to detect *ABCG2* gene expression (Supplementary Fig. S2*A*). ABCG2 was expressed at a low level in SP cells but not in NSP cells. Because it is responsible for the phenotype of Hoechst 33342 exclusion, we also measured ABCG2 protein levels on surface of SP and NSP cells. ABCG2 was expressed on ~45.0% of SP cells and only 1.5% of NSP cells (Supplementary Fig. S2*B* and *C*).

Tumor formation in NOD/SCID mice. We used the unsorted CNE-2 cell line for the preliminary experiment and choose 2×10^5 as the highest inoculation cell number for SP and NSP cells. Mice were euthanatized 4 or 9 weeks after inoculation, and the tumors were weighed and photographed. H&E staining was conducted to ensure that each mass was a tumor and not a connective tissue. The lowest inoculation cell number was 10,000, and mice formed tumors with SP but not NSP cells, which could only form tumors with 200,000 cells (Fig. 4*A* and *B*; Table 1). Pathology results confirmed that the tumors formed by SP and NSP cells were typical human NPC cells just like unsorted CNE-2 cells (Fig. 4*C* and *D*).

Immunofluorescence analysis of the expression of cytokines 8, 18, and 19 on SP and NSP cells. Because CSC cells lack distinct molecular markers, Hoechst 33342–dependent cell sorting is used as a substitute technique. Based on NPC characteristics, we investigated several squamous epithelium-related molecules, including cytokines 8, 18, and 19 to measure molecular marker

expression (Fig. 5). Immunofluorescence analysis revealed that cytokines 8 and 18 were strongly and equally expressed in the cytoplasm of SP and NSP cells, especially around the nucleus, and arranged in a mesh of bundles. However, cytokine 19 was almost negative in NSP cells and expressed at low levels in the cytoplasm of SP cells. We counted eight random fields and found that cytokine 19 was positive in ~14.5% of SP cells and only 5.0% of NSP cells ($P < 0.05$). This preliminary result suggested that cytokine 19 is a potential molecular marker that can aid in characterizing putative CSC.

Radiation and drug sensitivity differences between SP and NSP cells. We measured radiation sensitivity differences between SP and NSP cells. After radiation treatment, we cultured the cells for ~2 weeks until most cell clones had reached 50 cells, stained them with crystal violet, and counted the clone number. After exposure to X-ray (2 Gy), there were significant differences between the CFE of SP and NSP cells ($P < 0.05$; Fig. 6*A*). The CFE of SP cells was not significantly different before and after radiation ($P > 0.05$); however, there were significant differences between NSP cells before and after radiation ($P < 0.05$). These results showed that SP cells were more resistant to X-ray than NSP cells. As reported in pancreatic cancer (18), we also found that cyclopamine could also improve the radiation effect of SP cells CNE-2. Following exposure to 2 Gy of X-ray, cyclopamine decreased the CFE of SP cells from 33.1% to 19.8% ($P < 0.05$; Fig. 6*B*). Western blot revealed that SMO was expressed in SP cells but not in NSP cells and was reduced with each passage (Fig. 6*C*). Thus, the radiation sensitization effect may be related to the interaction between cyclopamine and SMO.

ABCG2 is considered a mitoxantrone resistance gene. Thus, we used mitoxantrone to further confirm this gene characteristic by drug resistance assay (Fig. 6*D*). Furthermore, we used verapamil to block the cell surface ABCG2 transporter and observe the toxicity changes of mitoxantrone. After treatment with mitoxantrone alone, SP cells showed strong resistance, whereas NSP cells were sensitive ($P < 0.001$). After pretreatment with verapamil, SP cells were also sensitive. The final IR of SP was enhanced and no longer significantly different from the IR of NSP cells ($P > 0.05$); however, the SP cells were significantly different without verapamil pretreatment ($P < 0.001$). No remarkable changes were detected in the IR of NSP cells in the presence or absence of verapamil ($P > 0.05$). These results indicated that the transporter of ABCG2 was responsible for the drug resistance of SP cells. We also conducted a drug sensitive assay with cisplatin [diamminedichloroplatinum(II)] and mitomycin C. We found that SP cells were much more resistant to diamminedichloroplatinum(II) than NSP cells ($P = 0.003$; Supplementary Fig. S3*A*) and slightly more resistant to mitomycin C ($P = 0.04$; Supplementary Fig. S3*B*).

Discussion

Although the 5-year SR for NPC has improved as a result of advances in radiation and chemotherapeutic strategies, the long-term prognosis is still poor. Thus, it is important to further elucidate the essence and origin of this cancer. It is well known that tumors are composed of heterogeneous cell types. Evidence suggests that tumors are a disease of stem cells and many, like normal organs, contain a small population of cells with a high proliferative capacity, self-renewing potential, multidifferentiation ability, and resistance to chemotherapy and radiotherapy. All these characteristics are similar to normal adult stem and even

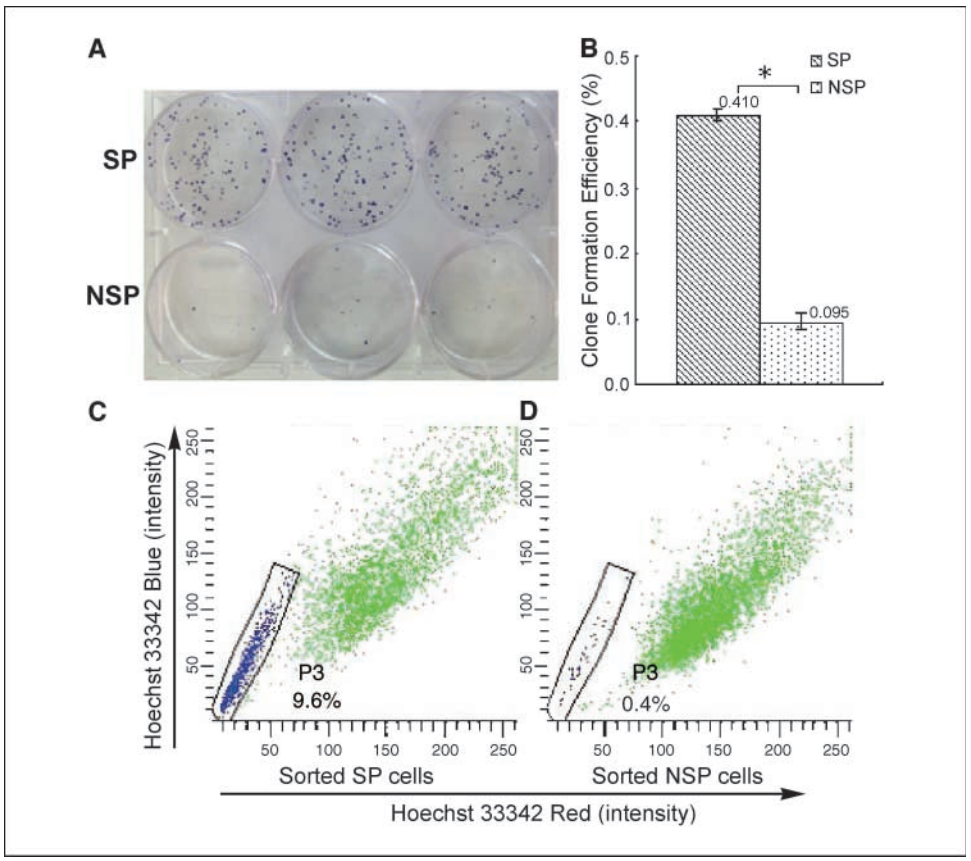

Figure 3. Clone formation and long-term culture differentiation of SP and NSP cells. *A* and *B*, clone formation results and statistical analysis. The CFE of freshly sorted SP cells was far stronger than NSP cells at an equal cell number. *, $P < 0.001$. *C* and *D*, 18 days after sorting, the SP cells contained ~9.6% SP cells, and the sorted NSP cells contained ~0.4% SP cells.

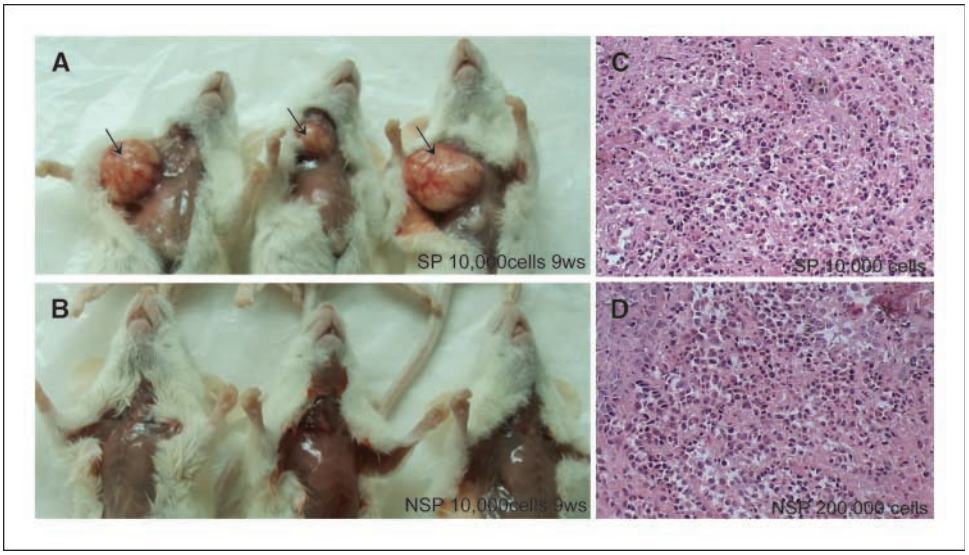

Figure 4. Tumor formation in NOD/SCID mice and H&E staining results. *A* and *B,* injection sites of NOD/SCID mice inoculated with 10,000 freshly sorted SP or NSP cells and euthanatized 9 wks later. All three mice formed tumors with SP cells but not with NSP cells. *C* and *D,* the H&E staining results of the tumors formed by SP cells (10,000) and NSP cells (200,000), respectively. Both were typical nasopharyngeal squamous cell carcinoma.

embryonic stem cells. Consequently, this subpopulation of cells is named CSC or tumor stem cells (TSC; refs. 21–23). Many normal tissues contain a few SP cells, including skeletal muscle (5), breast mammary epithelia (24), testicular tissue (25), and kidneys (26). They share many stem cell characteristics, including a long life span, relative quiescence, and resistance to drugs and toxins through ABC transporter expression. Compared with the bulk of nontumorigenic cancer cells, SP cells have a strong ability to form tumors after transplantation. Because they are resistant to chemotherapy and radiotherapy, they may contribute to tumor relapse even after most nontumorigenic cells are destroyed (27). CSC research on solid tumors has led to remarkable results in the past several years. In this study, we isolated SP cells from human NPC cell lines, helping to further characterize the biological properties of this cell type.

CNE-2 cells are the most poorly differentiated NPC cell line (28), containing ~2.6% SP cells within the usual SP cell range (11). The purity of the NSP cells was 96%, which was slightly poorer than the SP cells. Apparently, a few fluorescent negative SP cells may have attached to and been collected with the fluorescent positive NSP cells, decreasing the purity of this cell population. Even so, the NSP cell purity still met the experimental requirements.

Self-renewal and differentiation are properties of stem cells that allow them to generate additional CSC and phenotypically diverse cancer cells with a limited proliferative potential. *In vitro* experiments revealed that SP cells grow faster than NSP cells and have a higher CFE. In addition, *in vivo* treatment of NOD/SCID mice showed that 10,000 freshly sorted SP cells were required to form tumors, whereas at least 200,000 NSP cells were necessary. Thus, the tumor formation ability of SP cells was ~20 times higher than NSP cells. Asymmetrical cell division is a particularly attractive mechanism because it only requires one division for self-renewal and differentiation (29, 30). In long-term culture, SP cells may undergo asymmetrical cell division and differentiate into NSP cells, but NSP cells cannot reverse differentiate into SP cells. An early study by Scharenberg et al. (31) indicated that hematopoietic stem cells express high levels of the ABC drug transporter, ABCG2. However, this is turned off in most committed progenitor and mature blood cells. In our study, RT-PCR of ABCG2 revealed that although SP cells expressed ABCG2 at a low level, NSP cells failed to express it at all. Flow cytometric analysis also showed that the protein was higher on SP than NSP cells.

We also found that both SP and NSP cells had morphologic changes when they were maintained in UltraMEM (containing 5% FBS, 10 ng/mL bFGF, and EGF; Fig. 2*B*). Under these culture conditions, SP cells of CNE-2 lost their typical squamous cell shape and grew into fibroblast-like cells with long filaments that were no longer attached. It is known that during the epithelial-mesenchymal transition (EMT) process that occurs during embryonic development (32, 33), epithelial cells will obtain fibroblast-like properties, reduce intercellular adhesion, and acquire higher motility and invasiveness. We assume that SP and NSP cells underwent EMT because they more closely resembled fibroblasts than squamous epithelial cells. This conjecture requires further experiments to show whether cells obtain higher motility and invasiveness in UltraMEM.

Table 1. The *in vivo* tumor foundation ability of SP and NSP cells in NOD/SCID mice

	Cell no. (wks)					
	6×10^5 (4)	4×10^5 (4)	2×10^5 (4)	8×10^4 (4)	4×10^4 (4)	1×10^4 (4)
CNE-2	1/1	2/2	2/2	—	—	—
SP	—	—	3/3	3/3	2/3	3/3
NSP	—	—	2/3	0/3	0/3	0/3

According to the CSC theory, not all cancer cells are created equal (34). Tumors may have a built-in population of pluripotent cells that can survive chemotherapy and radiotherapy, and the resident CSC may repopulate the tumor even when the bulk of nontumorigenic cells are killed (35). The Hoechst 33342 exclusion ability conferred by ABC transporters forms the basis for the SP phenotype, and many chemical drugs may be pumped out of cells in the same way. Because they are the primary methods of treatment for NPC patients, we also conducted chemosensitivity and radiation sensitivity assays to investigate whether SP cells can resist treatment more readily than NSP cells. In the radiosensitivity assay, we found that SP cells were more tolerant to radiation than NSP cells following exposure to 2 Gy of X-ray. In the drug sensitivity assay, the SP cells were more resistant to mitoxantrone and this situation could be reversed by pretreatment with verapamil, showing that the ABCG2 transporter may be responsible for drug resistance (36, 37). Furthermore, freshly sorted SP cells are more resistant than NSP cells both to the common chemotherapeutic agent, diamminedichloroplatinum(II), and to the unfamiliar drug, mitomycin C, indicating that SP cells are widely resistant to chemotherapy.

In short, SP cells from the NPC CNE-2 cell line were more resistant to chemotherapy and radiotherapy than NSP cells. Our results support that SP cells isolated from CNE-2 have many stem cell properties, including unlimited proliferation potential, self-renewal, differentiation, resistance to chemotherapy and radiation, and strong tumor formation ability *in vivo*. These findings suggest that this may be a novel field for tumor research to us, helping to enhance many of our ideas about tumor formation and maintenance.

Identifying SP cells may also help us to further isolate CSC. Whereas SP cells possess many stem cell properties, particular cell surface molecular markers are required to distinguish kinds of CSC in different tissues from cancer cells with more limited proliferative potential. Al-Hajj et al. (1) suggested that breast cancer–initiating cells have a CD44(+)CD24(−/low)lineage(−) phenotype, whereas Singh et al. (2) confirmed that CD133 is the molecular marker for brain TSCs. Because all these molecules are related to tissue origin, we can learn from normal tissue stem cells and measure markers that are expressed in embryonic stem cells, hematopoietic stem cells, and epithelial stem cells. Microarray may help to identify molecules that distinguish between different mutations (27). Our data measuring differential expression of cytokine 19 showed that it was more highly expressed on fresh SP than NSP cells.

Second, this study might help us to determine the origin of tumors. For decades, tumor development has been regarded as a multistep process, in which progressive genetic alterations drive the transformation of normal human cells into highly malignant derivatives. CSC, by their very nature, arises as a consequence of genetic mutations. In some cases, CSC may arise from mutational transformation, in which normal stem cells gain a cancer phenotype, whereas in other cases, mutations that enhance self-renewal may cause committed progenitor cells or differentiated cells to adopt properties of CSCs (34, 38, 39). However, whether CSC comes from tissue special stem cells, differentiated progenitors, or mature cancer cells remains to be elucidated.

Third, and most importantly, this work should help us to design more effective strategies for tumor diagnosis and treatment. Despite advances in understanding tumor biology, treatment for cancers, including NPC, has not changed significantly in the past three decades. After isolating CSCs, we could determine their distinct properties in cell cycle regulation, signaling transduction, and so on. We know that several pathways that regulate normal stem cell self-renewal cause neoplastic proliferation when deregulated by

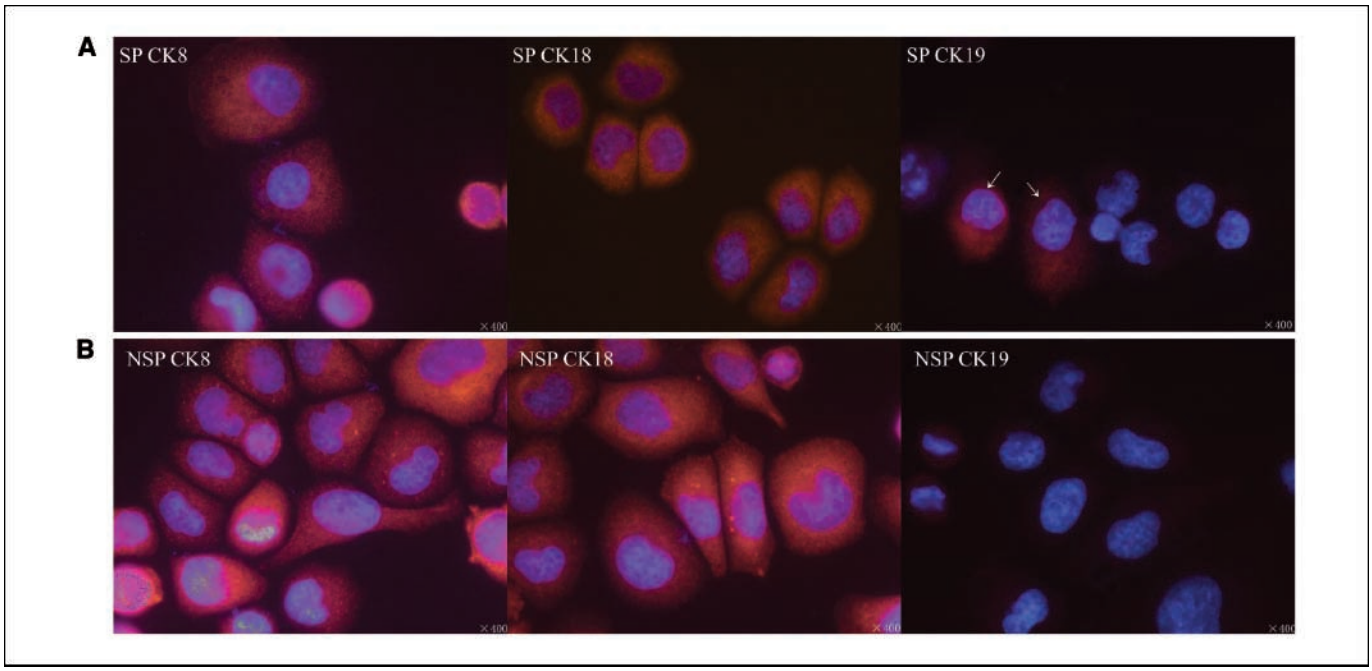

Figure 5. The expression of cytokines 8, 18, and 19 on SP and NSP cells. *A* and *B,* the expression of cytokines (*CK*) 8, 18, and 19 on freshly sorted SP and NSP cells, respectively. Cytokines 8 and 18 were highly expressed on both of them without statistically significant differences. However, cytokine 19 was only expressed at 14.5% (*arrows*) and 5.0% on SP and NSP cells, respectively ($P < 0.05$).

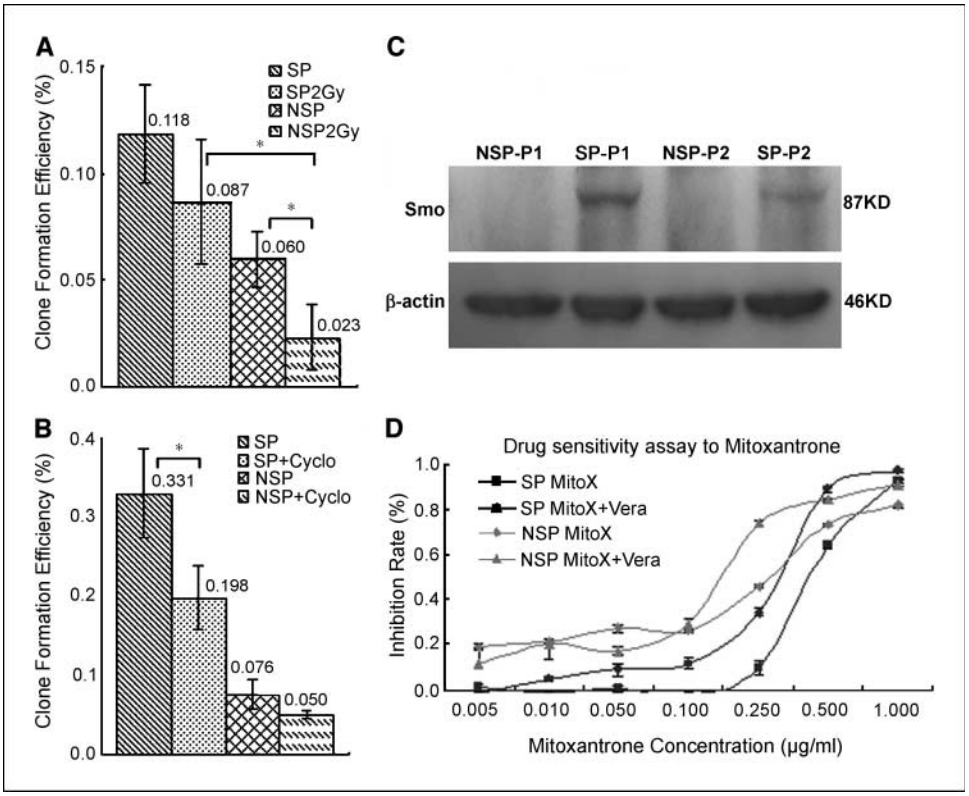

Figure 6. Results of radiation and chemotherapy drug sensitivity assays of SP and NSP cells and Western blot of SMO. *A,* significant differences were detected between SP2Gy and NSP2Gy and between NSP and NSP2G. *, $P < 0.05$. *B,* after preincubation with cyclopamine (*Cyclo*) for 36 h, the CFE of freshly sorted SP cells dropped from 33.1% to 19.8% following exposure to 2 Gy of X-ray. *, $P < 0.05$. However, there were no significant changes in the NSP cells with or without cyclopamine. *C,* Western blot results: protein SMO was expressed on SP cells (*lanes 2* and *4*) but not on NSP cells (*lanes 1* and *3*). The protein decreased on SP cells with each passage. *lane 2,* passage 1; *lane 4,* passage 2 after sorting. *D,* the sensitivity of newly sorted SP and NSP to mitoxantrone (*MitoX*). SP cells were more resistant to mitoxantrone than NSP cells ($P < 0.001$). The resistance could be reversed with verapamil pretreatment. *Columns* and *points,* mean of representative experiments done in triplicate; *bars,* SE.

mutations, such as WNT/β-catenin, SHH, Notch, and PTEN (34, 40). For example, the SHH pathway regulates epithelial-mesenchymal interactions, differentiation, and proliferation, stimulation of hedgehog signaling induces carcinogenesis and promotes cell survival in cancers of multiple organs. SMO is the signaling subunit of the SHH receptor and unregulated SMO signaling could trigger cell proliferation in skin in the absence of Ptch (41). Cyclopamine blocks the SHH signaling pathway by binding to SMO. In doing so, it blocks the oncogenic effects of SMO in fibroblasts, inhibits the growth of cells lacking Ptch function, arrests cell cycle at G_0-G_1, and induces apoptosis (42–44). Our finding that cyclopamine enhanced the radiation sensitivity of SP cells showed that SP cells may acquire some molecular changes involved in SHH signal transduction, and Western blot results on SMO also support this. A challenge of this approach will be to specifically target CSC without inducing toxicity in normal tissue stem cells (34, 45).

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