Isolation and characterization of cancer stem-like side population cells in human oral cancer cells

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Abstract: Recent studies suggest that cancer stem cells may be responsible for tumorigenesis and contribute to some individuals' resistance to cancer therapy. Some studies demonstrate that side population (SP) cells isolated from diverse cancer cell lines harbor stem cell-like properties; however, there are few reports examining the role of SP cells in human oral cancer. To determine whether human oral cancer cell lines contain a SP cell fraction, we first isolated SP cells by fluorescence activated cell sorting, followed by culturing in serum-free medium (SFM) using the SCC25 tongue cancer cell line, so that SP cells were able to be propagated to maintain the CSC property. Differential expression profile of stem cell markers (ABCG2, Oct-4 and EpCAM) was examined by RT-PCR in either SP cells or non-SP cells. Growth inhibition by 5-FU was determined by the MTT assay. Clonogenic ability was evaluated by colony formation assay. SCC25 cells contained 0.23% SP cells. The fraction of SP cells was available to grow in SFM cultures. SP cells showed higher mRNA expression of stem cell markers (ABCG2, Oct-4 and EpCAM) as compared with non-SP cells. Moreover, SP cells demonstrated more drug resistance to 5-FU, as compared with non-SP cells. The clone formation efficiency of SP cells was significantly higher than non-SP cells at an equal cell number ($P<0.01$). We isolated cancer stem-like SP cells from an oral cancer cell line. SP cells possessed the characteristics of cancer stem cells, chemoresistance, and high proliferation ability. Further characterization of cancer stem-like SP cells may provide new insights for novel therapeutic targets.

Keywords: cancer stem cells; side population; oral cancer; chemoresistance

Introduction

Although monoclonal in origin, most tumors appear to contain a heterogeneous population of cancer cells.[1] The concept of cancer stem cells (CSC) was introduced to explain this heterogeneity.[2,3] Recent studies suggest that CSC may be responsible for tumorigenesis and contribute to some individuals' resistance to cancer therapy.[2,3] Some studies demonstrate that side population (SP) cells isolated from diverse cancer cell lines harbor stem cell-like properties;[4-21] however, there are few reports examining the role of SP cells in human oral cancer.[5,6,15-18,20,21] Isolation of CSC-like SP cells from cancer cell lines has been successful using two distinct methods based on the properties of CSC. First, isolation of CSC is made possible by flow cytometry according to CSC characteristics.[7-9,14,16,17,20] Plotting fluorescence intensity on blue versus red wavelengths, the SP fraction appears as a low-fluorescent tail-shaped cell population. The SP phenotype is determined by the ability to efflux Hoechst 33342 dye through an ATP-binding cassette (ABC) membrane transporter. Second, the sphere formation of CSC is enriched under the cultivation of defined serum-free medium (SFM) with growth factors.[15,18,22,23] In these methods, however, the CSC population from the cancer cell line is not only small, but it is also difficult to maintain an enriched status of CSCs in long-term culture.[7,8,18,24,25] In our experiments, we first isolated SP cells by fluorescence activated cell sorting (FACS), followed by culturing in SFM containing basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), so that SP cells were able to be propagated to maintain the CSC property.

The purpose of this study was the characterization of CSC in the oral cancer cell line. The novel therapeutic strategies that selectively target the CSC subset might nonetheless achieve long-term disease eradication by exhausting self-renewal and growth potential of cancer tissues.

Materials and Methods

Cells

The human tongue cancer cell line SCC25, obtained from the American Type Culture Collection (Manassas, VA), was cultured in a 1:1 mixture of Ham's F-12/DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in the presence of 5% CO_2.

SP analysis and cell sorting

Cells were labeled with 2.5 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. The control cells were incubated in the presence of 50 μM verapamil (Sigma-Aldrich). Propidium iodine (PI) 1 μg/ml was added to discriminate dead cells. Analysis and sorting were performed on FACS Vantage SE (Becton Dickinson, San Jose, CA).

Sphere culture

After sorting, SP cells and non-SP cells of SCC25 were placed at a density of 1,000

cells/ml under stem cell conditions by resuspension in tumor sphere medium consisting of serum-free 1:1 mixture of Ham's F-12/DMEM, N_2 supplement (Invitrogen, Carlsbad, CA), 10 ng/ml human recombinant bFGF (Invitrogen), and 10 ng/ml EGF (Invitrogen), followed by culturing in ultra-low attachment plates (Corning, NY) for about 2 weeks.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIzol Reagent (Invitrogen) and first-strand cDNA was synthesized from 1 μg total RNA using Oligo d (T) primer (Invitrogen) and ReveTra Ace (TOYOBO, Osaka, Japan). For PCR analysis, cDNA was amplified by Taq DNA polymerase (TAKARA, Otsu, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous expression standard. Each PCR for cancer stem cell markers (*ABCG2*, *Oct-4* and *EpCAM*) and *GAPDH* was performed using PCR Thermal Cycler MP (TAKARA). Each primer was designed to encompass an exon junction to prevent templating from possibly contaminated genomic DNA. Primer sequences were, for *ABCG2*: F, AGC TGC AAG GAA AGA TCC AA and R, TCC AGA CAC ACC ACG GAT AA; for *Oct-4*: F, ATC CTG GGG GTT CTA TTT GG and R, CTC CAG GTT GCC TCT CAC TC; for *EpCAM*: F, CTG CCA AAT GTT TGG TGA TG and R, ACG CGT TGT GAT CTC CTT CT; and for *GAPDH*: F, ATG TCG TGG AGT CTA CTG GC and R, TGA CCT TGC CCA CAG CCT TG. The amplified products were separated by electrophoresis on ethidium bromide-stained 1.2% agarose gels. Band intensity was measured by Image J version 1.37.

Cell proliferation assay

The parental, SP or non-SP cells were seeded in the 96-well plate at a concentration of 5 $\times 10^3$ per well in DMEM/F-12 supplemented with 10% FBS. The cells were incubated for 24 h, followed by incubation for 0, 12, 24, 48, and 72 h. At each point, cells were incubated with 0.5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich). Four hours later, the medium was replaced with 100 μl dimethylsulfoxide (DMSO; Sigma-Aldrich) and vortexed for 10 min. Absorbance was then recorded at 570 nm using Easy Reader 340 AT (SLT-Lab Instruments, Salzburg, Austria), and drew the cell growth curve according to the data.

Colony formation assay

Parental, SP or non-SP cells were plated at about 200 cells per well in 6-well coated plates, and cultured in DMEM/F-12 supplemented with 10% FBS for 10 days. After most cell clones had increased to >50 cells, they were washed with PBS, fixed in methanol for 15 min, and stained with crystal violet for 15 min at room temperature. After washing out the dye, clones with >50 cells were counted as positive colonies. The percentage of cells that formed colonies is presented as clone formation efficiency (CFE).

5FU-IC_{50} and chemosensitivity assay

Parent SCC25 cells without sorting were seeded in the 96-well plate at a concentration of 5×10^3 per well in DMEM/F-12 supplemented with 10% FBS and allowed to attach overnight. The cells were treated with 5-FU (Sigma-Aldrich) at various concentrations. After 48 h, the number of surviving cells following 5-FU treatment was assessed using a MTT proliferation assay as described above. These experiments were performed in triplicate. 5FU-IC_{50} values (the drug concentration that corresponded to a reduction in cell survival of 50% compared with the survival of untreated control cells) were determined from dose response curves.

Parental, SP or non-SP cells were seeded in the 96-well plate at a concentration of 5×10^3 per well in DMEM/F-12 supplemented with 10% FBS. After 24 h, the cells were treated with the IC_{50} of 5-FU for 48 h. Cell viability (%) was determined by MTT assay, and calculated as a percentage of the absence of 5-FU.

Statistical analysis

Statistical analyses were performed using StatMate III (Atms Co., Tokyo, Japan). Continuous data are given as the mean ± standard deviation. Data sets were examined by one-way analysis of variance (ANOVA) followed by Scheffe's post-hoc test. $P < 0.05$ was considered significant.

Results

SP analysis

SCC25 Hoechst-low cells were sorted from the SCC25 cell line after excluding dead cells and cellular debris based on scatter signals and propidium iodide fluorescence. SP cells have been shown to exhibit a distinct projection pattern by actively effluxing Hoechst 33342 dye from cytoplasm. The SP cell fraction comprised 0.23% of the total cell population, but totally disappeared after treatment with the selective ABC transporter inhibitor Verapamil (Fig. 1).

Sphere formation

Isolated SP cells and non-SP cells of SCC25 were cultured in SFM. SP cells were able to grow, forming spheres at day 5 (Fig. 2a). Floating spheres in suspension generated from single cells of SCC25 increased in size over time (Fig. 2b); however, non-SP cells could not be propagated under stem cell conditions. Thus, after sorting, these cells were cultured and maintained in DMEM/F-12 supplemented with 10% FBS.

Expression of stem cell genes

To examine the expression difference of the stem cell genes between sphere forming SP and non-SP cells, the extracted RNA from parental, sphere forming SP or non-SP cells was analyzed by semiquantitative RT-PCR for *ABCG2*, *Oct-4* and *EpCAM*. The expression of *ABCG2*, *Oct-4* and *EpCAM* was significantly higher in sphere forming SP

cells than in parental and non-SP cells (Fig. 3).

Cell growth rate

The growth rate for each population was measured with an MTT assay for 6 days. SP cells had higher proliferative ability than that of parental and non-SP cells. On days 2, 3, and 4 after seeding, the growth rate of SP cells was significantly higher than that of parental and non-SP cells; however, after day 5, SP cells had reached a plateau. The growth rates were not significantly different between SP and non-SP cells (Fig. 4a).

Colony formation ability

Colony formation assays were repeated twice in triplicate. SP cells revealed significant increases in large colony formation ability compared with parental and non-SP cells (Fig. 4b and 4c).

5-FU chemosensitivity differences

The IC_{50} of 5-FU obtained from the dose response curve for parental SCC25 cells was 82.2 μM. SP cells were significantly more resistant to 5-FU than parental and non-SP cells (Fig. 5).

Discussion

Since the concept of CSC has been proposed to explain tumor cell heterogeneity, some research has suggested that current therapies fail to prevent cancer relapse and metastasis because of a small, surviving population of CSC.[2,3,24,25] It is assumed that the most effective therapy should target CSC. Recent researches on various solid tumors revealed the existence of CSCs, providing strong evidence for the presence of functional heterogeneity within the tumor population;[4,16,26] however, CSCs are very rare and their differentiated progeny are very inaccessible. The SP technique is the most widely used strategy to isolate CSCs from cancer cell culture.[7-9,14,16,17,20,21] Goodell et al. first examined the cell cycle distribution of whole bone marrow cells using Hoechst 33342 vital dye staining and discovered that the display of Hoechst fluorescence simultaneously at two emission wavelengths (blue 450 nm and red 675 nm) localizes a small, yet distinct unstained cell population that expresses stem cell markers ($Scal^{+}Lin^{neg/low}$).[27] SP cells are localized in the left lower quadrant of a FACS profile. Zhang et al. reported that the SP population was highly variable among the oral cancer cell lines, from as high as 10% of the cellular population to merely 0.2%;[16] however, other authors reported that the ratio of SP to non-SP cells at seven days post-culture in serum was almost the same as that before sorting.[8,28] On the other hand, some studies reported that SFM selection might be useful for CSC expansion.[15,29,30] Since serum causes irreversible differentiation of stem cells, SFM allows for maintenance of an undifferentiated stem cell status.[30] Moreover, the addition of EGF and bFGF has been

reported to induce the proliferation of CSC.[15,18] Kondo et al. reported that SP cells were isolated by FACS using Hoechst 33342 dye, and that their proportion increased by culturing in SFM with growth factors in the glioma cell line.[29] In this study, we isolated SP cells (0.23% of the total cell population) from the human tongue cell line SCC25. Moreover, the proportion of SP cells but not non-SP cells formed spheres, and was significantly increased by culturing in SFM with EGF and bFGF. Non-SP cells could not be propagated under stem cell conditions. These results suggest that SFM selection is capable of increasing SP cells from the tongue cancer cell line.

The cellular capacity of Hoechst 33342 dye efflux is determined by the concentration of ABC transporter superfamily efflux pumps in the plasma membrane, including multidrug resistance 1 (MDR1).[31,32] Human ABCG2 is the second member of the G subfamily of ABC transporters.[32] Elevated expression of ABCG2 has been observed in a number of putative CSCs from various cancers.[33-36] AGCG2 is a well-known marker useful for identifying and isolating CSCs. The *Oct-4* gene, a member of the POU family of transcription factors, was shown to be expressed in both embryonic and adult stem cells.[37] Oct-4 is involved in controlling not only the maintenance of embryonic stem cell pluripotency but also the proliferation potential.[37,38] Hu et al. reported that Oct-4 might maintain the survival of CSCs partly through the Oct-4/Tcl1/Akt1 pathway.[39] Moreover, Wang et al. demonstrated that the Oct-4/Tcl1/Akt1 pathway could affect cell survival and drug sensitivity by regulation of ABCG2;[40] therefore, Oct-4 is an important marker of CSCs. Epithelial adhesion

molecule (EpCAM) is also a CSC marker.[41-43] EpCAM is a glycosylated, 39-42 kDa, transmembrane protein.[44] Overexpression of EpCAM has been reported in various cancers, including colorectal cancer, tongue cancer and breast cancer.[44-47] Gonzalez et al. reported that EpCAM reduction resulted in quantitatively decreased proliferation and expression of stem markers, such as Oct-4, SSEA-1, and c-myc, and exogenous expression of EpCAM conversely compensated for the requirement of the stem cell phenotype.[48] In our study, all of these representative CSC markers (ABCG2, Oct-4, and EpCAM) were significantly increased in SP cells compared with non-SP cells; therefore, it is suggested that our isolated SP cells could characterize the properties of CSC.

Extensive proliferative and self-regeneration potential are fundamental characteristics of CSCs.[2,3] In vitro experiments revealed that SP cells grow faster than non-SP cells and have higher colony formation ability.[7,8,18,49] In our study, SP cells had higher proliferation rates and colony formation ability than non-SP cells in DMEM/F-12 supplemented with 10% FBS; however, after day 4, SP cells reached a plateau. After day 5, the growth rates were not significantly different among parental, SP, and non-SP cells. These results indicated that the ratio of SP to non-SP cells was almost the same as before sorting. The same phenomenon was previously reported by Kruger et al.[28] and supported the idea that tumorigenic cells are probably enriched in the SP fraction, but even non-SP cells may contain a small number of tumorigenic cells. On the other hand, it has been assumed that CSCs have a low rate of division and proliferation in restrictive niche environments;[3,24,25,50] however, SP cells were sorted from other tumor cells, might

separate from their niche environments, and then gain the ability to proliferate faster, in contrast to non-SP cells.[51] Consequently, we considered that SP cells were typical stem cells capable of self-regeneration, including high proliferative capacity and a colony formation potential greater than non-SP cells.

According to the CSC concept, chemotherapy kills most cells in a tumor; however, it is believed to leave CSCs behind, which might be an important mechanism in the development of resistance to chemotherapy. Indeed, CSCs are known to be significantly resistant to various chemotherapeutic agents *in vitro*.[7,8,18] Our results showed that SP cells were significantly more resistant to 5-FU than non-SP cells. The Hoechst 33342 exclusion ability conferred by ABC transporters forms the basis for SP phenotypes, and many chemical drugs may be pumped out of cells in the same way.[18] In our study, isolated SP cells showed a higher level of ABCG2 expression; therefore, it is suggested that the up-regulation of ABCG2 is closely associated with the resistance of SP cells to 5-FU.

In summary, we were able to isolate SP cells by FACS, followed by culturing in SFM. These SP cells are enriched as cells with CSC characteristics of self-regeneration, high proliferative capacity, and chemotherapy resistance. This characterization of CSCs may provide new insights into novel therapeutic targets for oral cancer treatment.

Conflicts of interest

None declared.

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References

1. Heppner GH, Miller BE. Tumor heterogeneity: biological implications and therapeutic consequences. *Cancer Metastasis Rev* 1983; **2**: 5-23.
2. Dalerba P, Cho RW, Clarke MF. Cancer stem cells: models and concepts. *Annu Rev Med* 2007; **58**: 267-84.
3. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008; **8**: 755-68.
4. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006; **444**: 756-60.
5. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, et al. Identification of a subpopulation of cells with cancer stem properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci USA* 2007; **104**: 973-8.
6. Harper LJ, Piper K, Common J, Fortune F, Mackenzie IC. Stem cell patterns in cell lines derived from head and neck squamous cell carcinoma. *J Oral Pathol Med* 2007; **36**: 594-603.
7. Wang J, Guo LP, Chen LZ, Zeng YX, Lu SH. Identification of cancer stem cell-like side population cells in human nasopharyngeal carcinoma cell line. *Cancer Res* 2007; **67**: 3716-24.
8. Sung JM, Cho HJ, Yi H, Lee CH, Kim HS, Kim DK, et al. Characterization of a stem cell population in lung cancer A549 cells. *Biochem Biophys Res Commun*

2008; **371**: 163-7.

9. Zhou J, Wang CY, Liu T, Wu B, Zhou F, Xiong JX, et al. Persistence of side population cells with high drug efflux capacity in pancreatic cancer. *World J Gastroenterol* 2008; **14**: 925-30.
10. Chiba T, Miyagi S, Saraya A, Aoki R, Seki A, Morita Y, et al. The polycomb gene product BMI1 contributes to the maintenance of tumor-initiating side population cells in hepatocellular carcinoma. *Caner Res* 2008; **68**: 7742-9.
11. Engelmann K, Shen H, Finn OJ. MCF7 side population cells with characteristics of cancer stem/progenitor cells express the tumor antigen MUC1. *Cancer Res* 2008; **68**: 2419-26.
12. Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. *Oncogene* 2008; **27**: 6120-30.
13. Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, et al. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res* 2008; **68**: 4311-20.
14. Loebinger MR, Giangreco A, Groot KR, Prichard L, Allen K, Simpson C, et al. Squamous cell cancers contain a side population of stem-like cells that are made chemosensitive by ABC transporter blockade. *Brit J Cancer* 2008; **98**: 380-7.
15. Chiou SH, Yu CC, Huang CY, Lin SC, Liu CJ, Tsai TH, et al. Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous

cell carcinoma. *Clin Cancer Res* 2008; **14**: 4085-95.

16. Zang P, Zhang Y, Mao L, Zhang Z, Chen W. Side population in oral squamous cell carcinoma possesses tumor stem cell phenotypes. *Cancer Lett* 2009; **277**: 227-34.
17. Yajima T, Ochiai H, Uchiyama T, Takano N, Shibahara T, Azuma T. Resistance to cytotoxic chemotherapy-induced apoptosis in side population cells of human oral squamous cell carcinoma cell line Ho-1-N-1. *Int J Oncol* 2009; **35**: 273-80.
18. Okamoto A, Chikamatsu K, Sakakura K, Hatsushika K, Takahashi G, Masuyama K. Expansion and characterization of cancer stem-like cells in squamous cell carcinoma of the head and neck. *Oral Oncol* 2009; **45**: 633-9.
19. Nishii T, Yashiro M, Shinto O, Sawada T, Ohira M, Hirakawa K. Cancer stem-like SP cells have a high adhesion ability to the peritoneum in gastric carcinoma. *Cancer Sci* 2009; **100**: 1397-402.
20. Song J, Chang I, Chen Z, Kang M, Wang CY. Characterization of side populations in HNSCC: highly invasive, chemoresistant and abnormal Wnt signaling. *PLos ONE* 2010; **5**: e11456.
21. Chen YC, Chen YW, Hsu HS, Tseng LM, Huang PI, Lu KH, et al. Aldehyde dehydrogenase 1 is a putative marker for cancer stem cells in head and neck squamous cancer. *Biochem Biophys Res Commun* 2009; **385**: 307-13.
22. Dey D, Saxena M, Paranjape AN, Krishnan V, Giraddi R, Kumar V, et al. Phenotypic and functional characterization of human mammary stem/progenitor cells in long term culture. *PLos ONE* 2009; **4**: e5329.

23. Bortolomai I, Canevari S, Facetti I, Cecco LD, Castellano G, Zacchetti A, et al. Development and critical characterization of a model derived from the A431 carcinoma cell line forming spheres in suspension. *Cell Cycle* 2010; **9**: 1194-206.

24. Graziano A, d'Aquino R, Tirino V, Desiderio V, Rossi A, Pirozzi G. The stem cell hypothesis in head and neck cancer. *J Cell Biochem* 2008; **103**: 408-12.

25. Prince MEP, Ailles LE. Cancer stem cells in head and neck squamous cell cancer. *J Clin Oncol* 2008; **26**: 2871-5.

26. Lim YC, Oh SY, Cha YY, Kim SH, Jin X, Kim H. Cancer stem cell traits in squamospheres derived from primary head and neck squamous cell carcinomas. *Oral Oncol* 2011; **47**: 83-91.

27. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 1996; **183**: 1797-806.

28. Kruger JA, Kaplan CD, Luo Y, Zhou H, Markowitz D, Xiang R,et al. Characterization of stem cell-like cancer cells in immune-competent mice. *Blood* 2006; **108**: 3906-12.

29. Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc Natl Acad Sci USA* 2004; **101**: 781-6.

30. Dou J, Gu N. Emerging strategies for the identification and targeting of cancer stem cells. *Tumor Biol* 2010; **31**: 243-53.

31. Schinkel A, Mayer U, Wagenaar E, Mol CAAM, van Deemter L, Smit JJM, et al. Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proc Natl Acad Sci USA* 1997; **94**: 4028-33.

32. Ding XW, Wu Jh, Jiang CP. ABCG2: a potential marker of stem cells and novel target in stem cell and cancer therapy. *Life Sci* 2010; **86**: 631-7.

33. Seigel GM, Campbell LM, Narayan M, Gonzalez-Fernandez F. Cancer stem cell characteristics in retinoblastoma. *Mol Vision* 2005; **11**: 729-37.

34. Ho MM, Ng AV, Lam S, Hung JY. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res* 2007; **67**: 4827-33.

35. Shi GM, Xu Y, Fan J, Zhou J, Yang XR, Qiu SJ, et al. Identification of side population cells in human hepatocellular carcinoma cell lines with stepwise metastatic potentials. *J Cancer Res Clin Oncol* 2008; **134**: 1155-63.

36. Wang YH, Li F, Luo B, Wang XH, Sun HC, Liu S, et al. A side population of cells from a human pancreatic carcinoma cell line harbors cancer stem characteristics. *Neoplasma* 2009; **56**: 371-8.

37. Campbell PA, Perez-Iratxeta C, Andrade-Navarro MA, Rudnicki MA. Oct4 targets regulatory nodes to modulate stem cell function. *PLos ONE* 2007; **2**: e553.

38. Matoba R, Niwa H, Masui S, Ohtsuka S, Carter MG, Sharov AA. Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling. PLos ONE 2006; 1: e26.

39. Hu T, Liu S, Breiter DR, Wang F, Tang Y, Sun S. Octamer 4 small interfering RNA

results in cancer stem cell-like cell apoptosis. *Cancer Res* 2008; **68**: 6533-40.

40. Wang XQ, Ongkeko WM, Chen L, Yang ZF, Lu P, Chen KK, et al. Octamer 4 (OCT4) mediates chemotherapeutic drug resistance in liver cancer cells through a potential Oct4-AKT-ATP-binding cassette G2 pathway. *Hepatol* 2010; **52**: 528-39.
41. Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, et al. Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci USA* 2007; **104**: 10158-63.
42. Munz M, Baeuerle PA, Gires O. The emerging role of EpCAM in cancer and stem signaling. *Cancer Res* 2009; **69**: 5627-9.
43. Kimura O, Takahashi T, Ishii N, Inoue Y, Ueno Y, Kogure T, et al. Characterization of the epithelial cell adhesion molecule $(EpCAM)^+$ cell population in hepatocellular carcinoma cell lines. *Cancer Sci* 2010; **101**: 2145-55.
44. Winter MJ, Nagtegaal ID, van Krieken JHJM, Litvinov SV. The epithelial cell adhesion molecule (Ep-CAM) as a morphoregulatory molecule is a tool in surgical pathology. *Am J Pathol* 2003; **163**: 2139-48.
45. Gastl G, Spizzo G, Obrist P, Dunser M, Mikuz G. Ep-CAM overexpression in breast cancer as a predictor of survival. *Lancet* 2000; **356**: 1981-2.
46. Went P, Vasel M, Bubendori L, Terracciano L, Tornillo L, Riede U, et al. Frequent high-level expression of the immunotherapeutic target Ep-CAM in colon, stomach, prostate and lung cancers. *Brit J Cancer* 2006; **94**: 128-35.
47. Yanamoto S, Kawasaki G, Yoshitomi I, Iwamoto T, Hirata K, Mizuno A.

Clinicopathologic significance of EpCAM expression in squamous cell carcinoma of the tongue and its possibility as a potential target for tongue cancer gene therapy. *Oral Oncol* 2007; **43**: 869-77.

48. Gonzalez B, Denzel S, Mack B, Conrad M, Gires O. EpCAM is involved in maintenance of the murine embryonic stem cell phenotype. *Stem Cell* 2009; **27**: 1782-91.
49. Wan G, Zhou L, Xie M, Chen H, Tian J. Characterization of side population cells from laryngeal cancer cell lines. Head Neck 2010; 32: 1302-9.
50. Borovski T, De Sousa E Melo F, Vermeulen L, Medema JP. Cancer stem cell niche: the place to be. Cancer Res 2011; 71: 634-9.
51. Burkert J, Wright NA, Alison MR. Stem cells and cancer. an intimate relationship. J Pathol 2006; 209: 287-97.

Figure legends

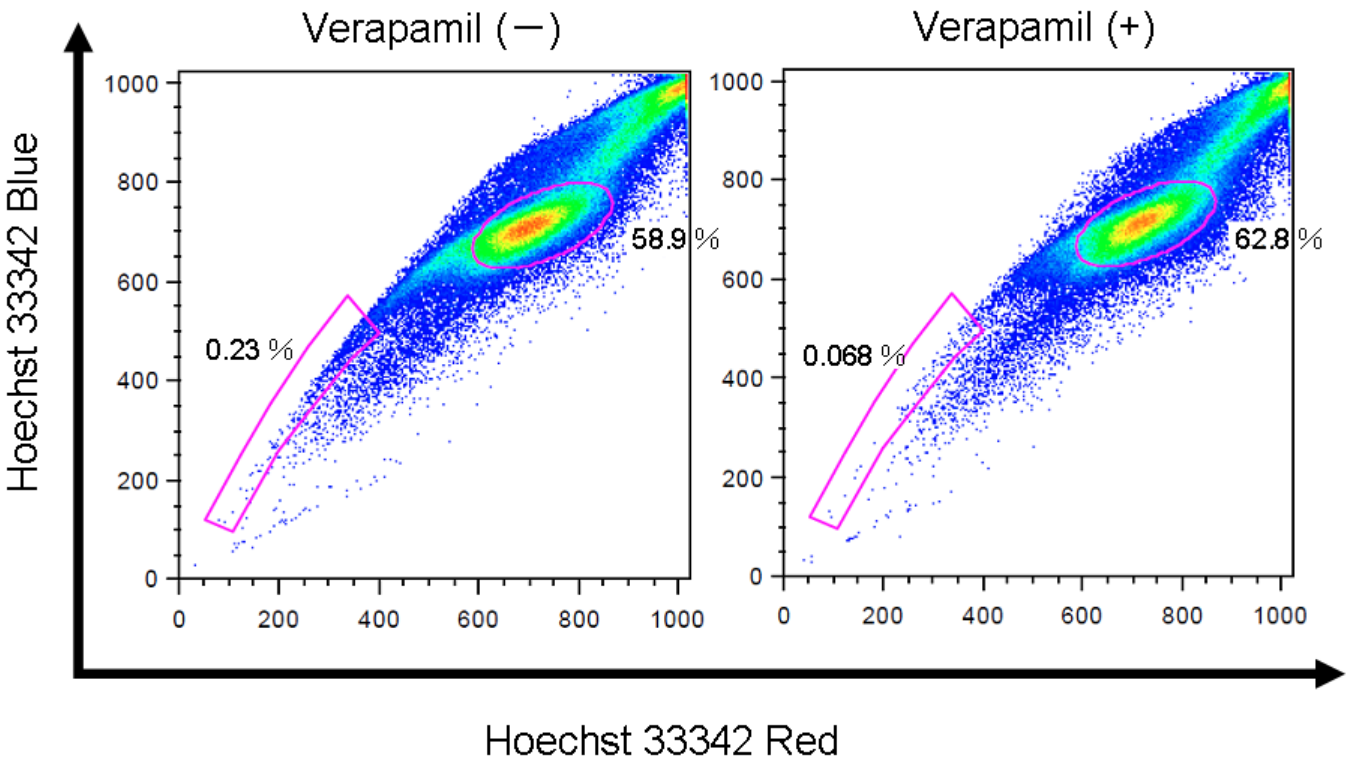

Figure 1 Identification of SP cells in oral cancer cell line SCC25. SCC25 cells were stained with Hoechst 33342 and then analyzed by flow cytometry in the absence or presence of verapamil. SP cells were gated and shown as a percentage. These experiments were performed in triplicate with similar results.

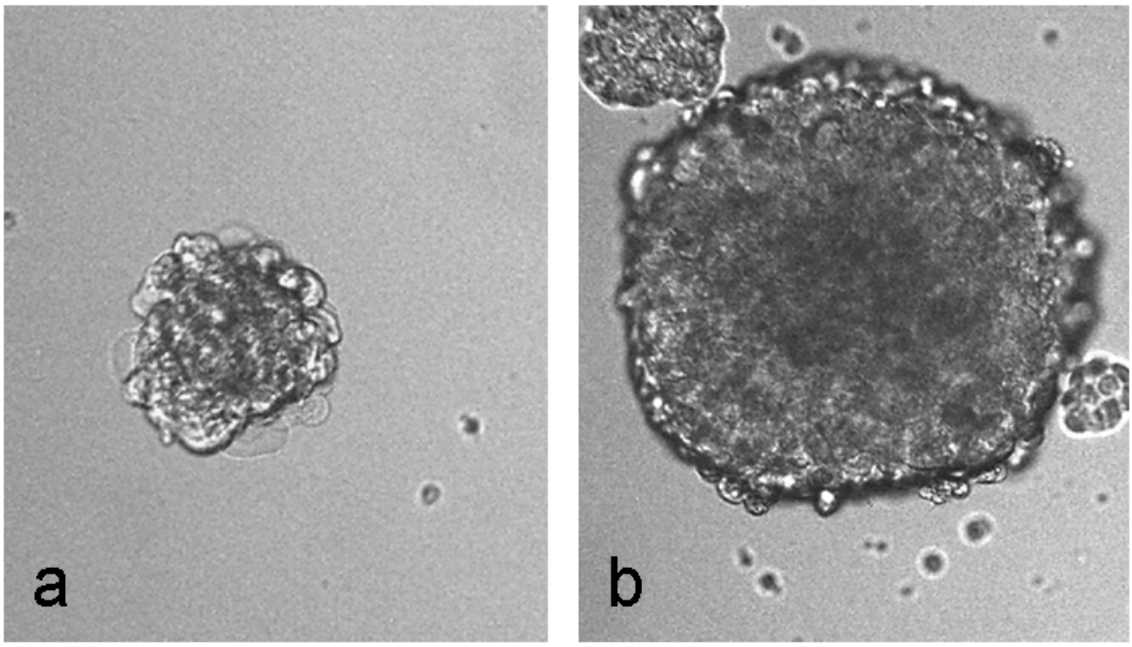

Figure 2 Representative phase contrast photomicrographs of tumor spheres from SP cells cultured in SFM supplemented EGF and bFGF. (a) SP cells were able to grow, forming spheres at day 5. (b) Spheres in suspension generated from single cells increased in size over time, imaged on day 14 of culture. (Original magnification, 200×).

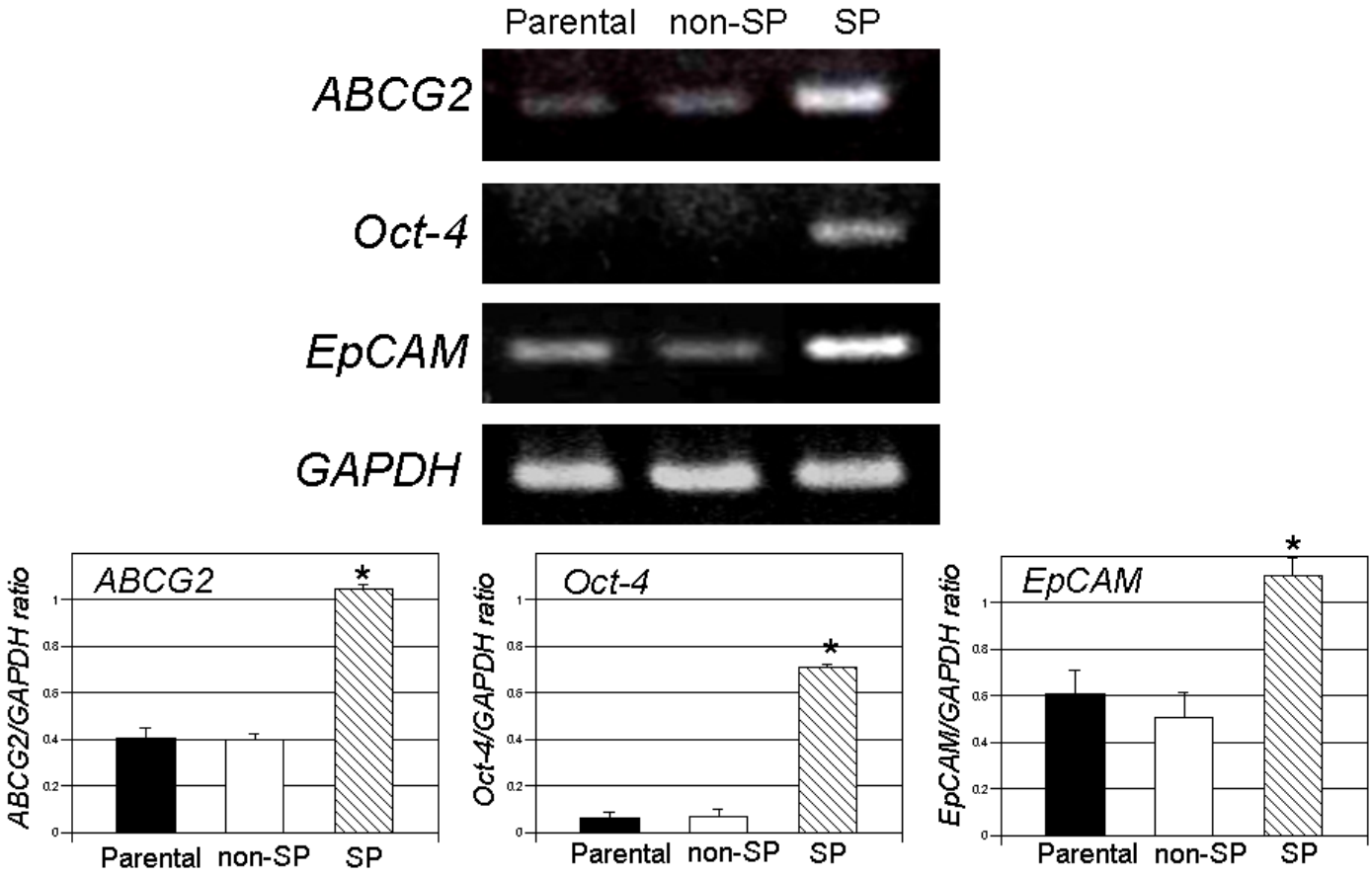

Figure 3 Expression of stem cell markers in SP and non-SP cells. The elevated expressions of ABCG2, Oct-4, and EpCAM genes in derived SCC25 were detected by RT-PCR. Data are presented as the means of three separate experiments, each performed in triplicate. Bars, SD. *P < 0.01, compared with parental and non-SP cells, respectively.

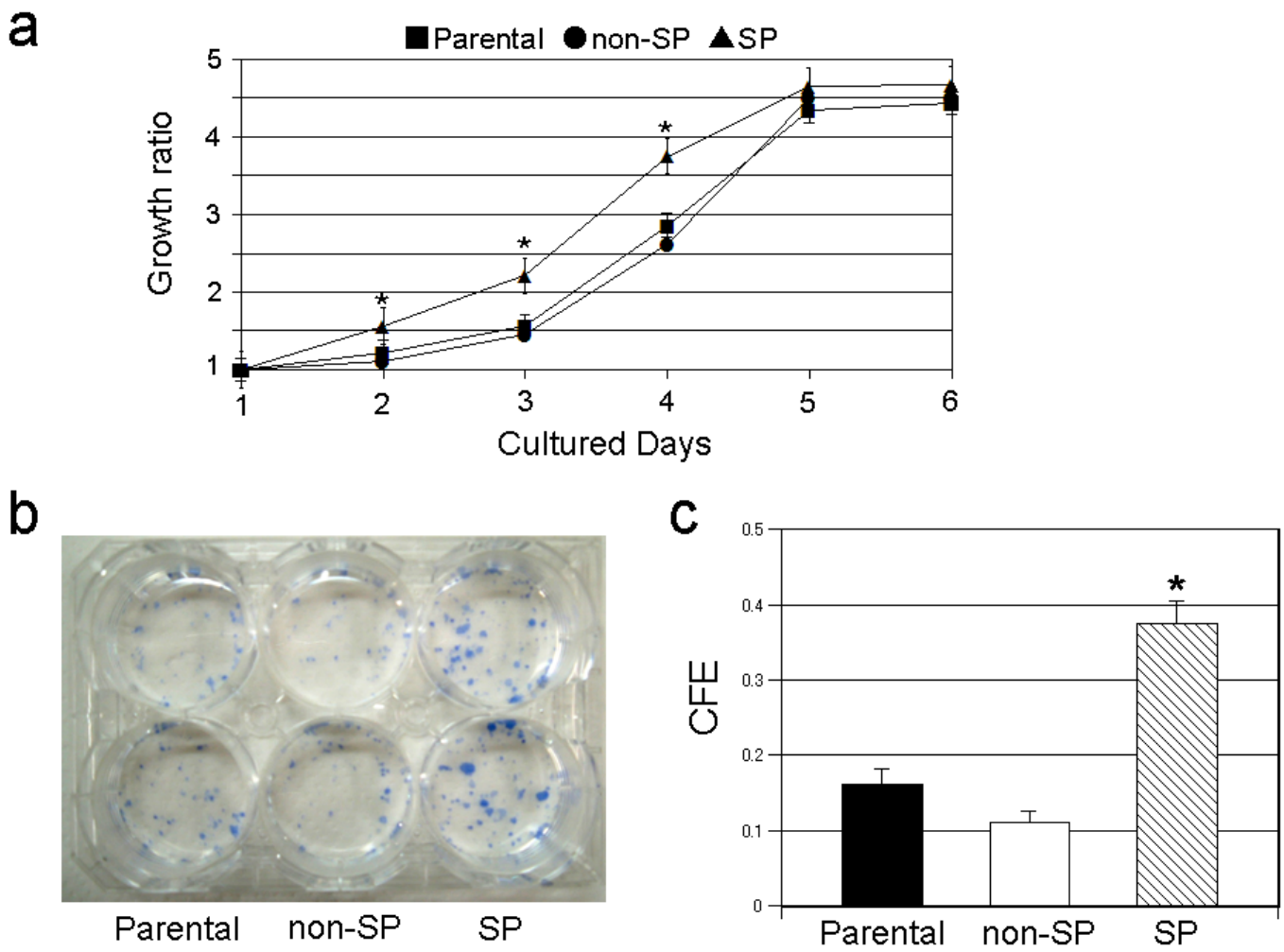

Figure 4 Cell growth curve and colony formation. (a) Growth curve of SP, non-SP, and parental cells cultured for 6 days. SP cells had higher proliferative ability than parental and non-SP cells. Data are presented as the means of three separate experiments, each performed in triplicate. Bars, SD. *P < 0.01, compared with parental and non-SP cells, respectively. (b and c) The clone formation efficiency (CFE) of SP cells was significantly higher than parental and non-SP cells with an equal cell number. The experiments were repeated twice in triplicate. Bars, SD. *P < 0.01, compared with parental and non-SP cells, respectively.

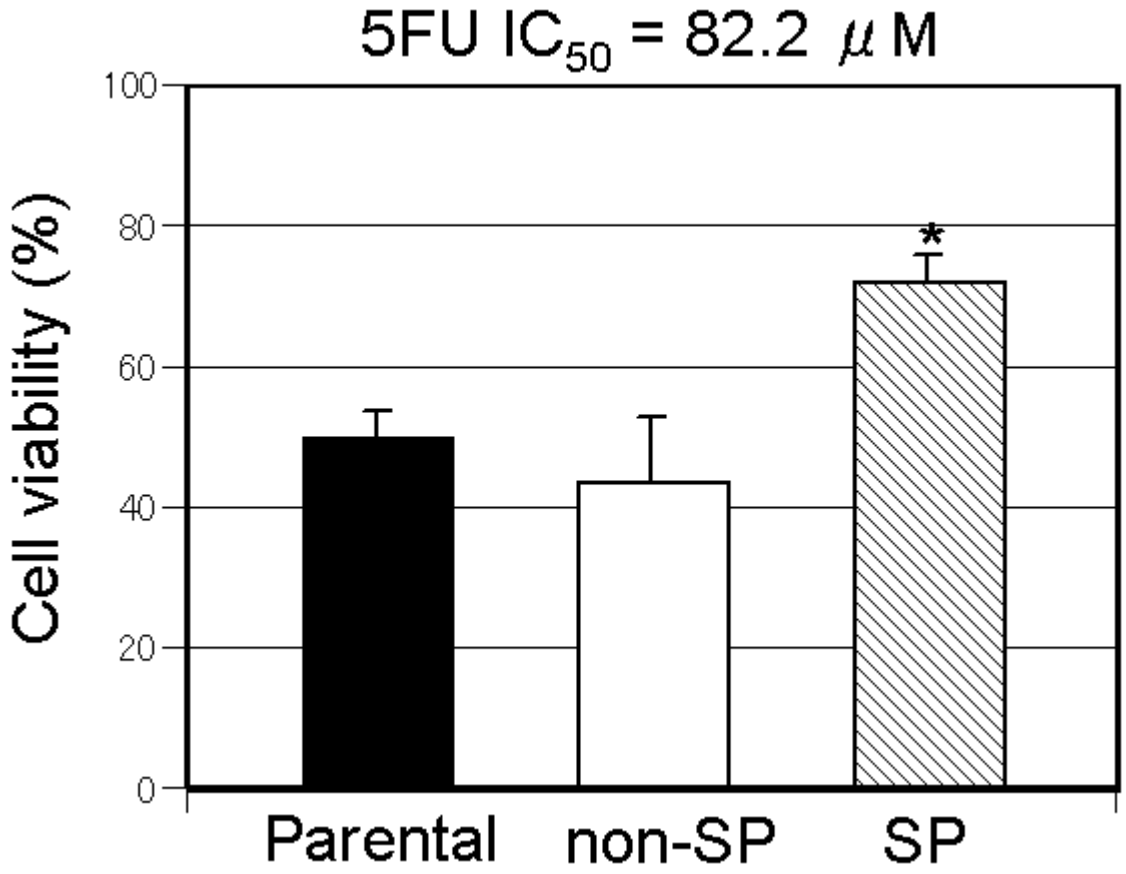

Figure 5 5-FU sensitivity in SP and non-SP cells derived from the SCC25 cell line. The cells were treated with the IC_{50} (82.2 μM) of 5-FU for 48 h. Data are presented as the means of three separate experiments, each performed in triplicate. Bars, SD. *P < 0.01, compared with parental and non-SP cells, respectively.