

# **Identification and Characterization of Cancer Stem Cells in Human Squamous Cell Carcinoma Using Side Population Analysis**

(Side population 法によるヒト扁平上皮癌がん幹細胞の同定及びその性質の検討)

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## ABSTRACT

Cancer stem cells are defined as a population having dual properties of self-renewal and multilineage differentiation potential, and continuously repopulate differentiated cancer cells. The self-renewal of normal stem cells is regulated by niche cells and that of cancer stem cells may also be regulated by the similar mechanism. We hypothesized that the cells at the border of a cancer nest of human squamous cell carcinoma may be cancer stem cells by their morphological similarity to normal stem cells. In order to demonstrate this hypothesis and examine whether the self-renewal of these cancer stem cells, if any, are regulated by surrounding stromal cells *in vivo*, we tried to identify and characterize cancer stem cells in human squamous cell carcinoma using side population (SP) analysis because SP cells have been reported to possess stem-cell-like properties in various normal tissues and cancers. A431 human squamous cell carcinoma cell line was able to recapitulate human squamous cell carcinoma phenotypes in SCID mice because its xenograft showed similar histology and expression property of Ki-67, cytokeratin 1, CD44, and integrin  $\beta 1$  to those of human squamous cell carcinoma. We demonstrated that cultured A431 cells contained 0.15-4 % of SP cells, which had tumorigenicity in SCID mice. Transplanted  $1 \times 10^5$  of SP cells formed a tumor bigger than the tumor formed by the same number of non-SP cells. Moreover,  $1 \times 10^4$  of SP cells formed a tumor by day 50, while the same quantity of non-SP cells did not (n=3). When  $3.3 \times 10^4$  of sorted SP cells, un-sorted cells, and non-SP cells were cultured, the total cell numbers of sorted SP cells were  $8.6 \times 10^4$  (day 5) and  $4.5 \times 10^5$  (day 7), those of un-sorted cells were  $5.1 \times 10^4$  (day 5) and  $2.2 \times 10^5$  (day 7), and those of non-SP

cells were  $2.0 \times 10^4$  (day 5) and  $1.3 \times 10^5$  (day 7), indicating that sorted SP cells proliferated the most rapidly. Furthermore, cultured SP cells generated both SP cells (4.38 %) and non-SP cells (95.62 %) by day 7, which suggested that sorted SP cells had a property of an asymmetric division. We also identified 3.25 % of SP cells in an A431 mouse xenograft. However, their character was different from that in cultured cells in expression property of integrin, which we thought of as a marker for cancer stem cells on the basis of immunohistochemical analysis. Whereas the SP cells in cultured A431 cells contained 99.4 % of integrin  $\beta 1$  (+) cells, the SP cells isolated from A431 xenograft contained 0.86 % of integrin  $\beta 1$  (+) cells. These results suggested that we should not consider the phenotype of the SP cells isolated from A431 xenograft (*in vivo*) as the same with that of the SP cells in cultured A431 cells (*in vitro*). It may be necessary to identify and characterize cancer stem cells *in vivo*, where they may interact with stromal cells, instead of analyzing cancer stem cells *in vitro*, in order to elucidate the regulation of self-renewal of cancer stem cells.

## INTRODUCTION

A fundamental problem in cancer research is identification of the cell type capable of initiating and sustaining the tumor growth *in vivo*. Conventional therapies may shrink tumors. However, some cancer cells survive after therapy and re-establish the tumor. The surviving cells may be cancer stem cells, and there is a report showing that glioma stem cell promotes radioresistance after ionizing radiation and the surviving cells are cancer stem cells (1), which supports this hypothesis. In order to explain the reason why tumor recurrence occurs, recent studies have attempted to prove the existence of cancer stem cells in various malignancies such as in leukemia (2) (3), breast cancer (4), brain tumor (5) and so on (6-8). The development of cancer stem cell-directed therapy may contribute to the eradication of the tumor cell compartment that survives against conventional therapies.

The concept of cancer stem cells is parallel to that of normal stem cells. Normal stem cells are defined as the cells having dual properties of self-renewal and multilineage differentiation potential, and continuously repopulate the mature cells of the organ system that they serve. Stem cells are also defined by their ability to divide asymmetrically. This division results in the formation of two daughter cells — one of which is another stem cell, and the other of which is a committed progenitor that is capable of further differentiation and proliferation but lacks the ability to self-renew. Cancer stem cells identified so far function in a similar way to initiate and sustain the growth of tumors while reproducing cancer stem cells and progenitor cells that make tumor cell population heterogeneous (9). Progenitor cells derived from cancer stem cells

seem to divide more rapidly than cancer stem cells do and this characteristic of proliferation may result in keeping cancer stem cells a small portion of tumor cell population.

In order to prevent cancer stem cells from disappearing, there seem to be a mechanism to maintain their self-renewal. Considering the similarity between normal stem cells and cancer stem cells, it seems likely that there is also at least some degree of overlap in the regulation of self-renewal in both types of stem cells. This possibility was raised by the fact that Notch signaling pathway, implicated to control self-renewal of stem cells, regulates number of both murine somatic and human embryonic stem cells (10) and its inhibition depletes cancer stem cells and blocks engraftment in embryonic brain tumors (11). In this way, the mechanism to maintain the self-renewal seems to be shared between both types of stem cells. The self-renewal of normal stem cells is maintained by niche cells (12). Niche cells provide an environment that isolates stem cells from differentiation, apoptotic, and other stimuli that would reduce stem cell reserves. There is little data available concerning the mechanism of self-renewal in normal or cancer stem cell populations and no research has yet been carried out to examine whether cancer stem cells require niche cells in order to maintain its self-renewal. If the self-renewal of cancer stem cells is maintained by niche cells, the destruction of the maintenance of cancer stem cells by impairing niche cells will be a fundamental therapy for cancer. We therefore, tried to identify and characterize cancer stem cells and the mechanism of their maintenance possibly regulated by niche cells.

Squamous cell carcinoma and normal epidermis, which is the normal counterpart

of squamous cell carcinoma, have similar morphological properties. In normal epidermis, stem cells exist in the basal layer and generate stem cells and progenitor cells through asymmetric division (13). These progenitor cells differentiate while moving toward the surface of epidermis and enter apoptosis (14). The proportion of the size of nuclei to that of cytoplasm decreases thorough differentiation (14). Squamous cell carcinoma also shows the same morphological change. Therefore, the cells located at the border, whose shape is similar to that of normal stem cells in epidermis, may be cancer stem cells.

In this study, our goal is to verify the hypothesis that cancer stem cells exist at the border of a nest of squamous cell carcinoma and their ability to self-renew may be maintained by surrounding stromal cells. This paper describes the results concerning identification and characterization of cancer stem cells in human squamous cell carcinoma. Recent studies have reported that normal tissue-specific stem cells such as those in hematopoietic system (15), mammary glands (16), and other tissues (17-22) and cancer stem cells from various tumors and cancer cell lines, such as neuroblastoma (23) and the C6 glioma cell line (9) are enriched in the population that efflux Hoechst 33342, called side population (SP). Therefore, the SP phenotype may reflect the properties of stem cells from various tissues and might be an appropriate candidate for putative cancer stem cells. We therefore, tried to define and characterize putative cancer stem cells in squamous cell carcinoma using SP analysis.

## **MATERIALS AND METHODS**

Animal care and experimental protocols were performed in accordance with procedures and guidelines established by the NCC Administrative Panels for Lab Animal Care. Severe Combined Immunodeficiency (SCID) mice were used in the experiments for xenograft formation of SP cells, non-SP cells, or un-sorted cells from cultured A431 cells and BALB/c *nu/nu* mice were used in the experiments for xenograft formation of ABCG2 (-) cells or un-sorted cells from cultured A431 cells.

### **Materials and Reagents**

The human squamous cell carcinoma cell line A431 was obtained from the RIKEN BioResource Center (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) and 1 % penicillin and streptomycin and incubated at 37°C in an atmosphere containing 5 % CO<sub>2</sub>. DMEM, penicillin and streptomycin were purchased from SIGMA Chemical (St Luis, MO). C.B-17/*lcr-scid/scid*/Jcl mice and BALB/cAJcl-*nu/nu* mice were obtained from CLEA Japan, Inc (Tokyo, Japan). Anti-integrin  $\alpha 6$  (CD49f) and integrin  $\beta 1$  (CD29) were purchased from R & D SYSTEMS, Inc. (Minneapolis, USA). Anti-Cytokeratin1 and CD-44 were purchased from Novocastra Laboratories (Newcastle, United Kingdom). Anti-integrin  $\beta 4$  (CD104) for immunohistochemistry was purchased from Abcam (Cambridge, United Kingdom). Anti-Ki-67 and Envision (labeled Polymer-HRP Anti-mouse kit) were obtained from DakoCytomation (Fort Collins, CO). Hanks' Balanced Salt Solution (HBSS) and HEPES were purchased from Invitrogen Life

Technologies (Carlsbad, CA). Hoechst 33342 and Propidium iodide were obtained from EMD Biosciences, Inc (Darmstadt, Germany). Verapamil was purchased from SIGMA. Collagenase Type I was purchased from Wako (Osaka, Japan). Anti-ABCG2, Purified Mouse IgG1, Fluorescein isothiocyanate (FITC) conjugated mouse IgG1, phycoerythrin (PE) conjugated mouse IgG1 and polyclonal rabbit anti-mouse immunoglobulins/FITC were obtained from eBioscience (San Diego, USA). Anti-integrin  $\alpha$  2 (CD49b),  $\alpha$  3 (CD49c) and  $\alpha$  V (CD51) were purchased from IMMUNOTECH (Marseille, France). Anti-integrin  $\alpha$  6 (CD49f) and  $\beta$  1 (CD29) were purchased from CHEMICON (Temecula, USA). Anti- integrin  $\beta$  4 (CD104) for flow cytometry was purchased from BD Pharmingen, (Franklin Lakes, NJ).

### **Human Oral Epidermis and Oral Squamous Cell Carcinoma**

After obtaining informed consent, four cases of oral squamous cell carcinomas were obtained from subjects at National Cancer Center Hospital East. Normal tissues resected with carcinoma were also used for H & E staining and immunohistochemistry.

### **Immunohistochemistry**

Immunohistochemistry of tumor subjects and A431 xenograft was performed to evaluate the expression of integrin  $\beta$  1, integrin  $\beta$  4 and integrin  $\alpha$  6 using frozen sections and to evaluate the expression of Ki-67, cytokeratin 1, and CD44 using paraffin-embedded section. Frozen sections were fixed in 10 % formalin neutral buffer solution at room temperature for 1 minute. The endogenous peroxidases were quenched

with 0.3 % H<sub>2</sub>O<sub>2</sub> in phosphate-buffered-saline (PBS). Anti-integrin  $\beta$  1 (clone 4B7R), integrin  $\beta$  4 (clone 450-9D), and integrin  $\alpha$  6 (clone MAB1778) was applied and incubated for 1 hour at room temperature, followed by incubation with Envision (labeled Polymer-HRP Anti-mouse kit) for 30 minutes at room temperature. Staining without adding anti-human first antibodies was performed for a control in each sample. Samples were finally incubated with diaminobenzidine peroxidase substrate to give a brown stain and counterstained with hematoxylin. The samples were mounted with cover slips. When paraffin-embedded sections were stained, sections were deparaffinised and heated in the recommended unmasking solution at 95 °C for 20 minutes before quenching of endogenous peroxidases.

### **SP Analysis: Hoechst 333442 Exclusion Assay and Fluorescence-Activated Cell**

#### **Sorting for SP**

Hoechst 33342 exclusion assay and FACS for SP were performed according to a previously described method for isolating SP cells for hematopoietic stem cells using Hoechst 33342, which is a DNA binding dye that allows identification of cell cycle. Cultured A431 cells and the cells from A431 xenograft were used for the Hoechst 33342 exclusion assay to identify SP cells. FACS for SP was performed using cultured A431 cells but not A431 xenograft cells because of the limited number of the cells that were available for sorting. The SP cells were characterized by low blue and low red fluorescence intensity on a dot-plot displaying dual wavelength of Hoechst blue versus red. Briefly, the cells were suspended at  $1 \times 10^6$  cells/ml in HBSS supplemented with

3% FBS and 10 mM HEPES. These cells were then incubated at 37°C for 90 minutes with 7.5 µg/ml Hoechst 33342. The cell suspension was added to cold PBS and centrifuged and resuspended in cold HBSS containing PI (2µg/ml) to identify dead cells. PI-positive dead cells were excluded from the analysis. Cells were filtered through 40-µm cell strainer. To identify SP, 50µM of verapamil for cultured A431 cells, and 500µM for the cells from A431 xenograft was pre-added before staining for Hoechst 33342. To ensure that the SP cells were sorted, a sorting gate was chosen in which the inhibition of Hoechst exclusion by verapamil was highest. The non-SP cells were sorted from cells that were in the G0/G1 stage of cell cycle. All of the PI-negative cells were sorted as sham-sorted cells. To ensure that sorting was performed precisely, a small part of the sorted cells were re-SP analyzed and we confirmed that sorted non-SP cells did not contain SP cells. Sorted cells were used for immunodeficient xenograft transplantation, proliferation assay and re-SP analysis. These experiments were performed at FACSVantageSE fluorescence-activated cell sorter (Becton Dickinson) by using a dual-wavelength analysis (blue; 424-444 nm; and red; 675 nm). Hoechst 33342 and PI dyes were excited with 350 nm UV and 488 nm argon lasers, respectively. The 424/44 and 675/20 band-pass filters, in combination with a 640 long-pass dichroic filter, were used for detection of Hoechst blue and red emission, respectively. At least 50,000 events were collected before analysis. All flow cytometric data were analyzed with CELLQuest software (Becton Dickinson). Cells were displayed in a Hoechst blue versus Hoechst red dot-plot to visualize the SP.

### **Xenograft Digestion**

Xenograft was dissected into small fragments, further minced with a sterile scalpel, and then placed in a solution of DMEM containing 10mg/ml Collagenase Type I . The mixture was incubate at 37°C for up to 10 h to allow complete digestion. Cells were filtered through 40- $\mu$ m cell strainer, and then stained for flow cytometry analysis.

### **Flow Cytometry Analysis with Various mAb Staining and FACS with ABCG2 mAb Staining**

The cells from cultured A431 were used for integrin  $\alpha$  2, integrin  $\alpha$  3, integrin  $\alpha$  V, integrin  $\alpha$  6, integrin  $\beta$  1 and integrin  $\beta$  4 staining analysis, and ABCG2 staining analysis and cell sorting by flow cytometry. For staining, we used anti-integrin  $\alpha$  2 (clone Gi9), integrin  $\alpha$  3 (clone C3(VLA3), integrin  $\alpha$  V (clone AMF7), FITC conjugated anti-integrin  $\alpha$  6 (clone CBL45 F) and PE conjugated anti-integrin  $\beta$  1 (clone TDM29) and anti-integrin  $\beta$  4 (clone 439-9B). For staining analysis and cell sorting, we used a functional grade purified anti-human ABCG2 mAb (clone 5D3). This antibody reacts with the extracellular portion of the human ABCG2 protein and suitable for flow cytometry analysis of living cells. Briefly, cells from cultured A431 at  $1 \times 10^6$  cells/100 ml were incubated with mAb antibodies (dilution of each mAb antibody was subjected to the manufacture's protocols) for 15 minutes on ice, washed with PBS/1% FBS three times, incubated with FITC conjugated goat anti-mouse secondary antibody for 15 minutes on ice, washed with PBS/1% FBS three times, and then resuspended in PBS/3 % FBS/0.02 % EDTA. The cells were kept on ice until flow

cytometry was performed. Instead of the mAb, samples were incubated with mouse IgG1 for isotype control. When the cells were used for integrin  $\alpha 6$ , integrin  $\beta 1$  or integrin  $\beta 4$  staining, cells from cultured A431 cells at  $1 \times 10^6$  cells/100 ml were incubated with mAb antibodies (dilution of each mAb antibody was subjected to the manufacture's protocols) for 15 minutes on ice, washed with PBS/1% FBS three times and kept on ice until flow cytometry was performed. For integrin  $\alpha 6$  mAb, FITC conjugated mouse IgG1 was used as an isotype control, and for integrin  $\beta 1$  or  $\beta 4$  mAb, PE conjugated mouse IgG1 was used. Fluorescence-activated cell sorting (FACS) was performed using FACSCalibur (Becton Dickinson). A 488-nm argon laser excited both FITC and PE and band-pass filters of 530/15 and 585/21 were used to measure emitted light, respectively. Gates in the forward scatter versus side scatter diagrams were used to exclude debris. At least 10,000 events were collected before analysis. All flow cytometric data was analyzed with CELLQuest (Becton Dickinson). We performed the sorting of two subpopulations differing in their expressions of ABCG2. The cells except for debris were sorted as sham-sorted cells. The sorted ABCG2 (-) cells and sham-sorted cells were used for immunodeficient xenograft transplantation.

### **Flow Cytometry Analysis for SP with mAb Staining**

Cultured A431 cells and the cells from A431 xenograft were used for flow cytometry analysis for SP with mAb staining. Hoechst 33342 exclusion assay was performed according to the method described above. After the staining of Hoechst 33342, the cells were suspended in PBS/3 % FBS/0.02 % EDTA and the mixture was used for mAb

staining in accordance with the method described above. Anti-ABCG2 (clone 5D3), PE conjugated anti-integrin  $\beta$  1 (clone TDM29) were used for staining.

### **Proliferation Assay and Re-SP Analysis of FACS-Sorted Cells**

FACS-sorted SP cells, non-SP cells and sham-sorted cells were cultured in DMEM, supplemented with 10% FBS, 1 % penicillin and streptomycin. At day 0, day 5 and day 7, cultured cells were counted by trypan blue exclusion method and used for Hoechst 33342 exclusion assay and flow cytometry analysis for SP in order to measure the percentage of SP cells in each culture. Each population was seeded, in triplicate, at  $3.3 \times 10^4$  cells onto 24-well culture plate.

### **Doxorubicin Survival Assay**

A431 cells were seeded at  $7.5 \times 10^5$  cells onto 10 cm culture plate in DMEM/10% FBS /1 % penicillin and streptomycin. Doxorubicin was added to the medium at 0  $\mu$ g/ml, 0.1 $\mu$ g/ml and 1 $\mu$ g/ml of final concentration 96 h after the seeding of A431 cells. Medium containing doxorubicin at each concentration was exchanged in two days. At day 1, day 3 and day 5, the numbers of surviving cells were counted by trypan blue exclusion method and these cells were used to Hoechst 33342 exclusion assay and flow cytometry analysis for SP in order to measure the percentage of SP cells in surviving cells against doxorubicin.

### **Immunodeficient Xenograft Transplantation**

Cultured A431 cells or FACS-sorted cells were suspended in a volume of 200  $\mu$ l of DMEM medium. The SCID mice were then injected s.c. on the flank with SP cells, non-SP cells, or sham-sorted cells and the nude mice were injected s.c. on the flank with ABCG2 (-) cells or sham-sorted cells. At day 50, SCID mice were sacrificed and the tumor volume was measured. Up to 12 weeks, tumor formation in nude mice was observed.

## **RESULTS**

### **Histology and Immunohistochemical Analysis of Normal Oral Epidermis, Squamous Cell Carcinoma and an A431 xenograft**

In order to examine whether the phenotype of human squamous cell carcinoma resembles that of human normal epidermis, and whether an A431 xenograft could be an appropriate model reproducing squamous cell carcinoma, H & E staining and immunohistochemistry were performed. H & E staining of normal oral epidermis (Fig. 1A1 and A2), of differentiated cell carcinoma (Fig. 1B1 and B2) and of an A431 xenograft (Fig. 1C1 and C2) showed that these tissues demonstrated the change of morphology from a basal layer toward an apical layer. The change of morphology was a decrease of the proportion of the size of nuclei to that of cytoplasm. Immunohistochemical staining for Ki-67, a marker for proliferation, were performed (Fig. 1D-F), showing that all three tissues expressed Ki-67 only in certain basal or suprabasal layer cells, two or three layers more differentiated than basal layers. Immunohistochemical staining for cytokeratin 1 were performed (Fig. 1G-I). Cytokeratin 1, a keratinocyte differentiation marker, was expressed in the cytoplasm of the suprabasal layers and in the apical layers but not in the basal layers in each tissue. Immunohistochemical staining for CD44 were performed (Fig. 1J-L). CD44 is known to be a breast epithelium stem cell marker (24). CD44 was expressed in apical, suprabasal and basal layers but strongly expressed in basal layers in all three tissues. H & E staining and immunohistochemical staining showed that the most primitive cells in squamous cell carcinoma localized at the border of its nest and were closed to

surrounding stromal cells whereas more differentiated tumor cells were not closed to them. Then, we also performed immunohistochemical staining for several adhesion molecules; integrin  $\beta$  1, integrin  $\beta$  4, and integrin  $\alpha$  6, which were reported to express in stem cells in normal epidermis (25-27). Immunohistochemical staining for integrin  $\beta$  1 (Fig. 1M-O), staining for integrin  $\beta$  4 (Fig. 1P-R), and staining for integrin  $\alpha$  6 (Fig. 1S-U) were performed, showing that integrin  $\beta$  1 was expressed in both suprabasal and basal layers but strongly expressed in basal layers in A431 xenograft and expressed in basal layers in normal epidermis and squamous cell carcinoma. Integrin  $\alpha$  6 and integrin  $\beta$  4 were expressed only in the basal layers in A431 xenograft but hardly expressed in normal epidermis and squamous cell carcinoma. These data suggested that A431 cells recapitulated the tumor in SCID mice and that CD44 and integrin  $\beta$  1, strongly expressed in the basal layers of all three tissues, may be an appropriate marker for the border cells whose phenotype may be similar to normal stem cells.

### **SP Detection from Cultured A431 Cells and Characterization of Its Tumorigenicity and Proliferation Activity**

We performed Hoechst 33342 exclusion assay and flow cytometry analysis for the detection and characterization of SP cells in cultured A431 cells because we considered that the SP phenotype was likely to be an appropriate marker for cancer stem cells. Flow cytometry analysis of cultured A431 cells showed the existence of SP cells (Fig. 2A1, Region 1 (R1)) which effluxed the Hoechst 33342 dye. Dye efflux from A431 SP

cells was inhibited in the presence of 50  $\mu$ M verapamil (Fig. 2A2), evidenced by decreased SP cells from 0.50% (Fig. 2A1) to 0.03% (Fig. 2A2). Next, A431 SP cells were sorted from R1 (Fig. 2A1), and non-SP A431 cells were sorted from Region 2 (R2) in (Fig. 2A1) for the characterization of SP cells. All cells alive (PI (-) cells) were sorted as sham-sorted cells. In order to examine whether SP cells from cultured A431 cells satisfied the definition of cancer stem cells, the tumorigenicity of the SP cells in SCID mice was investigated. SP cells and non-SP cells were injected into the flank of SCID mice. At day 50, SCID mice were sacrificed for the measurement of tumor volume (Fig. 2B) and H & E staining of the xenograft formed by SP cells or by non-SP cells were performed (Fig. 2C1 and C2). The volume of the tumor formed by SP cells and non-SP cells showed greater tumorigenicity of SP cells than non-SP cells when  $1 \times 10^5$  cells were injected. Moreover,  $1 \times 10^4$  of SP cells formed a tumor by day 50, while the same quantity of non-SP cells did not (n=3). H & E staining of the xenograft demonstrated cytologic and architectural features similar to the parental A431 xenograft (Fig. 2C1, 2C2, 1C1, and 1C2). To examine the ability to divide in an asymmetric manner and proliferation property,  $3.3 \times 10^4$  of FACS-sorted SP cells, non-SP cells, and sham-sorted cells were cultured. The cell numbers and the proportion of SP cells in each culture were measured at day 5 and day 7 (Fig. 2D). The total cell numbers of sorted SP cells were  $8.6 \times 10^4$  (day 5) and  $4.5 \times 10^5$  (day 7), those of sham-sorted cells were  $5.1 \times 10^4$  (day 5) and  $2.2 \times 10^5$  (day 7), and those of non-SP cells were  $2.0 \times 10^4$  (day 5) and  $1.3 \times 10^5$  (day 7), indicating that sorted SP cells proliferated the most rapidly. Furthermore, cultured SP cells generated both SP cells (4.38 %) and

non-SP cells (95.62 %) by day 7, which suggested that sorted SP cells had a property of an asymmetric division. Non-SP cells also generated both SP cells (7.26 %) and non-SP cells (92.74 %) by day 7. When the proliferation of sorted cells was examined, the percentage of the SP cells in sorted SP cells decreased from 88.48 % (day 0) to 4.38% (day 7), indicating that non-SP cells generated from SP cells proliferated rapidly and contributed to the growth of the whole cell population. Non-SP cells generated SP cells and non-SP cells *in vitro* and formed a tumor in SCID mice, indicating that non-SP cells may contain cancer stem cells.

#### **SP Cells in Cultured A431 Cells Seemed to Have a Resistance to Doxorubicin**

In order to characterize the SP cells, a survival assay against doxorubicin, a famous anti-cancer drug, was performed. A431 cells were seeded at  $7.5 \times 10^5$  cells onto 10 cm culture plate in DMEM/10% FBS/1% penicillin and streptomycin. Doxorubicin was added to the medium at 0  $\mu\text{g/ml}$ , 0.1 $\mu\text{g/ml}$  and 1 $\mu\text{g/ml}$  of final concentration. At day 1, day 3, and day 5, the numbers of surviving cells were counted and these cells were used to measure the percentage of the SP cells in surviving cells against doxorubicin. The decrease in the number of surviving cells was dependent on the concentration of doxorubicin and on the exposure time (Fig.3A). The percentage of the SP cells in the total surviving cells increased depending on the concentration of doxorubicin (Fig. 3B), suggesting that SP cells in squamous cell carcinoma may have higher drug resistance than non-SP cells.

## **ABCG2 Expression of Cultured A431 Cells, Its Correlation to SP Phenotype and Its Tumorigenicity**

We tried to find the surface marker whose expression reflects the SP phenotype because purification by using surface marker is easier than FACS-sorting of SP cells. Because recent studies have suggested that the ATP-Binding Cassette protein, ABCG2 is one of a molecular determinant of the SP phenotype(28), we examined the expression of ABCG2 in cultured A431 cells and its correlation to the SP phenotype. Single-cell suspension from cultured A431 cells was used for flow cytometry analysis for SP combined with ABCG2 mAb staining. Flow cytometry analysis of cultured A431 cells showed a distinctive small population of ABCG2 (+) cells (5.17 %) (Fig. 3C). Flow cytometry analysis of cultured A431 cells showed the existence of SP cells (0.15 %) (Fig. 3D) inhibited in the presence of 50  $\mu$ M verapamil, evidenced by decrease to 0.02% (data not shown). For an experimental control, SP analysis without mAb staining was also performed and the same percent of SP cells were detected (0.15 %). However, when cultured A431 cells were used for SP analysis before (Fig. 2A), they contained more SP cells (0.50 %), indicating that there was the range of concentration of SP cells in cultured A431 cells. ABCG2 (-) cells were sorted, and all A431 cells alive (PI (-) cells) were sorted as sham-sorted cells. Flow cytometry analysis for SP was performed using ABCG2 (-) cells (Fig. 3E1) or ABCG2 (+) cells (Fig. 3E2), showing that ABCG2 (-) cells contained 0.06 % of SP cells but ABCG2 (+) cells did not. Consequently, there was no correlation between ABCG2 expression and the SP phenotype. To examine whether the expression of ABCG2 is responsible for tumorigenicity, ABCG2 (-) cells or

sham-sorted cells were injected into BALB/cA<sup>Jcl-nu/nu</sup> mice. ABCG2 (+) cells were not sorted because of the limited number for sorting. When  $1 \times 10^6$  cells were injected, both ABCG2 (-) cells and sham-sorted cells formed a tumor. Less than  $1 \times 10^5$  ABCG2 (-) cells did not form any tumor, nor did sham-sorted cells (Table. 1). Because there was no correlation between the expression of ABCG2 and the SP phenotype in A431 cell line, and this expression was not a determinant of tumorigenicity, the expression of ABCG2 may not be a substitution for the SP phenotype.

### **SP Phenotype and Its Correlation to Integrin Expression in the Cells from A431 Xenograft**

In order to examine the relation between SP cells and expression of integrin  $\beta 1$ , which we thought of as a marker for border cells of a cancer nest, single-cell suspension from A431 xenograft was used for flow cytometry analysis for SP combined with  $\beta 1$  mAb staining. Flow cytometry analysis of cells from A431 xenograft showed a distinctive population of integrin  $\beta 1$  (+) cells (12.69 %) (Fig. 4A1, A2). Flow cytometry analysis of the cells from A431 xenograft demonstrated 3.25 % of SP cells (Fig. 4B1). Dye efflux from the A431 SP cells was inhibited in the presence of 500  $\mu$ M verapamil, evidenced by decreased SP from 3.25% in (Fig. 4B1) to 0.24% in (Fig. 4B2). Flow cytometry analysis for SP cells was performed using integrin  $\beta 1$  (+) cells (Fig. 4C1) and integrin  $\beta 1$  (-) cells (Fig. 4C2) showed that integrin  $\beta 1$  (+) cells contained 0.29 % of SP cells while integrin  $\beta 1$  (-) cells contained more SP cells (3.21 %). The

percentage of the SP cells in integrin  $\beta 1$  (+) cells was lower than that of the whole cells from A431 xenograft, indicating that SP cells were more enriched in integrin  $\beta 1$  (-) cells in A431 xenograft. The SP cells in cultured A431 cells contained high percentage of integrin  $\beta 1$  (-) cells (99.4 %) (data not shown). However, the SP cells in the cells from A431 xenograft contained low percentage of integrin  $\beta 1$  (+) cells (0.86 %) (data not shown). These contradictory results indicated that the SP cells *in vitro* may differ from the SP cells *in vivo* in this cell line.

#### **Flow Cytometry Analysis with Various mAb Staining of Cultured A431 *in Vitro* and Cells from A431 Xenograft *in Vivo***

Cultured A431 cells may differ from the cells from A431 xenograft in other integrin-expression property. Single cells from cultured A431 and from A431 xenograft were used for integrin  $\alpha 2$ , integrin  $\alpha 3$ , integrin  $\alpha 6$ , integrin  $\alpha V$ , integrin  $\beta 1$  and integrin  $\beta 4$  staining analysis. Flow cytometry analysis revealed that integrin  $\alpha 2$  (99.6 %, 45.2 %), integrin  $\alpha 3$  (99.7 %, 68.2 %), integrin  $\alpha 6$  (98.8 %, 25.7 %), integrin  $\alpha V$  (99.0%, 59.2%), integrin  $\beta 1$  (99.4 %, 12.69 %) and integrin  $\beta 4$  (90.0 %, 25.6 %) were expressed in cultured A431 cells and the cells from A431 xenograft, respectively (Table 2). The percentages of integrin-expressing cells in cultured A431 cells were higher than the cells from A431 xenograft. These results indicate that cultured A431 cells differ from the cells from A431 xenograft in several integrin-expression properties, suggesting that the SP cells in cultured A431 cells and the SP cells from A431 xenograft may have more differences in addition to the

expression of integrin  $\beta 1$ .

## DISCUSSION

### **Human Squamous Cell Carcinoma Showed Normal Epidermis-Like Morphological Change Thorough Differentiation and Recapitulated the Phenotype of the Initial Tumor**

In order to examine both the morphological similarity between normal epidermis and differentiated squamous cell carcinoma, and validity of A431 mouse xenograft as a model of human squamous cell carcinoma, H & E staining and immunohistochemistry were performed. H & E staining and immunohistochemical staining for Ki-67 which is a marker of proliferating cell, for Cytokeratin1 which is a differentiation marker, and for adhesion molecules which are stem cell markers in normal epidermis, indicated that oral squamous cell carcinoma and A431 xenograft showed similar morphological change and expression properties through differentiation. This demonstrated that A431 cells, established from human squamous cell carcinoma, may recapitulate human squamous cell carcinoma, which indicates that A431 xenograft is applicable as a model of human squamous cell carcinoma. There was also a similarity in expression of CD44 and integrin  $\beta 1$  that are often expressed in normal stem cells (24-27), between normal epidermal stem cells and the cells at the border of a cancer nest. Moreover, the biological similarity between normal stem cells (29) and cancer stem cells (5) has been reported. On these grounds, we consider that the border cells are cancer stem cells and that the expression of CD44 and integrin  $\beta 1$  may be a marker for cancer stem cells in squamous cell carcinoma. It may be possible to enrich the putative cancer stem cells using these surface markers followed by further analysis.

### **Cancer Stem Cells were Enriched in SP Cells Which Grew and Differentiated on the Basis of Cancer-Stem-Cell-Model**

We performed Hoechst 33342 exclusion assay and flow cytometry analysis for the detection of SP in cultured A431 cells and FACS-sorting of the SP cells for characterization of them. Flow cytometry analysis of cultured A431 cells showed the existence of SP cells. To examine whether SP cells sorted from cultured A431 satisfied the definition of cancer stem cells, the tumorigenicity in SCID mice of the SP cells was investigated. Tumor volume of the SP cells and non-SP cells at day 50 revealed that SP cells had greater tumorigenicity than non-SP cells. H & E staining of xenograft from the SP cells demonstrated the cytologic and architectural features similar to human squamous cell carcinoma. In addition to the activity of SP cells to repopulate the tumor, whether they divided asymmetrically was examined. FACS-sorted SP cells produced both SP cells and non-SP cells. Sorted SP cells proliferated the most rapidly. However, the percentage of the SP cells decreased through proliferation. These results indicated that the SP cells in cultured A431 cells had both a greater potential of tumorigenicity and ability to divide asymmetrically, while non SP-cells produced by SP-cells grew fast to contribute to the growth of tumor population and to keep SP cells a small portion. These suggestions support the hypothesis that cancer stem cells of A431 cells are enriched in SP cells because SP cells satisfied the cancer-stem-cell-model very well. However, Re-SP analysis of sorted cells showed that non-SP cells produced both SP cells and non-SP cells. It may be because the contaminated SP cells in sorted non-SP cells

produced both SP cells and non-SP cells, or it may be because non-SP cells as well as SP cells divide asymmetrically to produce both SP cells and non-SP cells, indicating that the SP phenotype is reversible. Besides, xenograft formed by non-SP cells also showed the same histology with human squamous cell carcinoma, indicating that non-SP cells could also recapitulate the tumor. On these grounds, we considered that cancer stem cells do not exist only in SP cells and non-SP cells may also contain cancer stem cells *in vitro*.

When the proliferation of sorted SP cells and sham-sorted cells was examined, sorted SP cells proliferated to show 4.38 % of SP cells in the culture at day 7. The percentage of the SP cells in non-sorted A431 cells was 4% at day 0, rose to 24.7 % at day 5, but decreased to 4.15 % at day 7, which was close to the initial percentage although the total number of the cells increased about four times. Interestingly, sorted SP cells and non-sorted cells showed the similar percentage of the SP cells at day 7, indicating that A431 cell population required an appropriate percentage of SP cells to maintain its homeostasis *in vitro*.

### **SP Cells Resisted Doxorubicin**

In order to characterize these SP cells, the survival assay against doxorubicin was performed. Surviving A431 cells cultured in the presence of doxorubicin at 0  $\mu\text{g/ml}$ , 0.1 $\mu\text{g/ml}$  and 1 $\mu\text{g/ml}$  were counted and the percentage of SP cells in these cells was analyzed. The number of surviving cells decreased dependently on the concentration of doxorubicin and on the exposure time. However, the percentage of SP cells in surviving

cells increased depending on the concentration of doxorubicin, suggesting that the cells which remain after therapies and re-establish tumor might be SP cells. We plan to examine whether sorted SP cells in A431 cells will provide high drug efflux capacity of doxorubicin in comparison with non-SP cells.

### **ABCG2 Expression Was not Correlated to SP Phenotype and Did not Determine the Ability to Form a New Tumor**

Trying to find more proper marker for easier purification of cancer stem cells than the SP phenotype, we considered the expression of ABCG2, a member of membrane efflux pumps of the ATP-binding cassette (ABC) transporter superfamily, because recent studies have suggested that ABCG2 is a molecular determinant of the SP phenotype (28). SP cells preferentially express ABCG2 (23,30-35) and absent or low levels of SP cells have been observed in *Abcg2* KO mice in the lungs (30), skeletal muscle and bone marrow (36). Enforced expression of these membrane transporters with retroviral vector was shown to have direct functional effects on murine stem cells (28). Moreover, the high drug efflux capacity of some cancer SP cells correlated with strong expression of the ABCG2 and ABCA3 (23). Therefore, we examined the expression of ABCG2 in cultured A431 cells and its correlation to the SP phenotype. Flow cytometry analysis of cultured A431 cells showed a distinctive small population of ABCG2 (+) cells and SP cells. However, ABCG2 (+) cells did not contain SP cells but ABCG2 (-) cells did. It may be reasonable to consider that the SP phenotype of A431 cells, the ability to efflux, may be regulated by other many transporters (23,35,37,38).

To examine whether the expression of ABCG2 is responsible for tumorigenicity, nude mice were injected with ABCG2 (-) cells or sham-sorted cells. Two distinct populations in ABCG2 expression showed no difference in tumorigenicity, indicating that the expression of ABCG2 was not responsible for tumorigenicity of A431 cells. This result agrees with the report that ABCG2 (+) and ABCG2 (-) cancer cells are similarly tumorigenic in human glioma cell line, prostate cancer cell line, and breast cancer cell line (39), while does not agree with the report that ABCG2 is a marker for cancer stem cells in retinoblastoma (40). The contribution of ABCG2-expression to tumorigenicity may differ between various cancers. In A431 cells, ABCG2-expression may not be an appropriate marker for their cancer stem cells.

#### **SP Cells in Cultured A431 Expressed Integrin but SP Cells in A431 xenograft Did not Express Integrin**

Cultured A431 cells differed from the cells from A431 xenograft in several integrin-expressing properties, indicating that each cell population may be regulated differently by surrounding environment, which means that dealing with putative cancer stem cells in A431 xenograft, where stromal cells exist, may be more appropriate than dealing with those in cultured A431 cells, in order to elucidate their maintenance by niche cells. To identify the cancer stem cells in A431 xenograft, we hypothesized that cancer stem cells would be localized at the border of a nest of squamous cell carcinoma from their morphological similarity to normal epidermal stem cells. Identification of the molecular properties of the border cells, especially the surface markers, will make

possible to purify the putative cancer stem cells using the surface marker from A431 xenograft for their characterization. Therefore, we performed immunohistochemical analysis of A431 mouse xenograft, indicating that CD44 and integrin  $\beta 1$  may be a good marker for the border of the cells that resembles normal stem cells. Therefore, we examined whether the distinct population expressing integrin  $\beta 1$  would be detectable by flow cytometry analysis and whether there would be any correlation between the expression of integrin and the SP phenotype *in vivo*. Flow cytometry detected the SP cells and the expression of  $\beta 1$ . The SP cells in the cells from A431 xenograft contained 0.86 % of integrin  $\beta 1$  (+) cells. However, SP cells in cultured A431 contained more integrin  $\beta 1$  (+) cells (99.4 %), indicating that the SP cells *in vitro* may differ from the SP cells *in vivo* in this cell line. Flow cytometry analysis using the cells from A431 xenograft also showed that integrin  $\beta 1$  (+) cells did not contain SP cells and integrin  $\beta 1$  (-) cells contained both SP cells and non-SP cells, indicating that the expression of integrin  $\beta 1$  was not correlated to the SP phenotype *in vivo*.

The first possibility is that using the SP phenotype as the marker of cancer stem cells *in vivo* as well as *in vitro* may be proper but considering integrin expression may not be appropriate. The second possibility is that the properties of the SP cells *in vivo* are different from those of the SP cells *in vitro*, and integrin-expressing properties, not the SP phenotype, reflect the cancer stem cells properties *in vivo*. In order to determine whether using the SP phenotype or integrin expression is suitable to define cancer stem cells as a marker *in vivo*, it is necessary to analyze the tumorigenicity and the activity of repopulate the tumor using the cells isolated by each marker. Then, we will try to

construct the proper experimental model enabling for us to examine the regulation of cancer stem cells by niche cells.

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## FIGURE LEGENDS

**Fig. 1.** H & E (A1, A2, B1, B2, C1, and C2), and immunohistochemical staining (D-X) in human normal oral epidermis (A1, A2, D, G, J, M, P, S, V), human oral squamous cell carcinoma (B1, B2, E, H, K, N, Q, T, W), and A431 xenograft (C1, C2, F, I, L, O, R, U, X). All three tissues expressed Ki-67 only in certain basal or suprabasal layer cells (D-F). Cytokeratin 1 was expressed in the cytoplasm of the suprabasal layers and the apical layers but not in the basal layers in each tissue (G-I). CD44 was expressed in both suprabasal and basal layers but strongly expressed in the basal layers in all three tissues (J-L). Integrin  $\beta$  1 was expressed only in the basal layers in normal epidermis (M), and expressed in both basal layer and suprabasal layers but strongly expressed in the basal layers in squamous cell carcinoma (N), and in A431 xenograft (O). Integrin  $\beta$  4 was not expressed in normal epidermis (P) and not expressed in squamous cell carcinoma (Q), and expressed only in the basal layers in A431 xenograft (R). Integrin  $\alpha$  6 was not expressed in normal epidermis (S) and not expressed in squamous cell carcinoma (T), and expressed only in the basal layers in A431 xenograft (U). Staining without adding anti-human first antibodies was performed for a control in each tissue (V-X).

**Fig. 2. (A1):** Flow cytometry analysis of cultured A431 cells showing SP (R1) which effluxed the Hoechst 33342 dye. A431 SP cells were sorted from R1, and non-SP A431 cells were sorted from R2. All living cells (PI-negative cells) were sorted as sham-sorted cells. **(A2):** Dye efflux from A431 SP cells was inhibited in the presence of

50  $\mu$ M verapamil, evidenced by decreased SP from 0.50% in **(A1)** to 0.03% in **(A2)**. **(B)**: The tumor volume formed by the injection of SP cells and non-SP cells showed greater tumorigenicity of SP cells than non-SP cells when  $1 \times 10^5$  and  $1 \times 10^4$  cells were injected. **(C)**: H & E staining in xenograft formed by the injection of sorted SP cells **(C1)** and sorted non-SP cells **(C2)** showing the similar morphology to human squamous cell carcinoma. **(D)**: The cell numbers of cultured FACS-sorted SP cells, non-SP cells and sham-sorted cells showing that sorted SP cells proliferated most rapidly, followed by sham-sorted and non-SP cells, and that the percentage of SP cells in sorted-SP cells decreased through proliferation.

**Fig. 3. (A)**: Doxorubicin killed the cultured A431 depending on the concentration of doxorubicin and on the exposure time. **(B)**: The percentage of SP cells in surviving cells depended highly on the concentration of doxorubicin. **(C1, C2)**: Flow cytometry analysis of cultured A431 showed a distinctive small population of ABCG2 (+) cells (5.17 %). **(D)** Flow cytometry analysis of cultured A431 cells showing SP cells which effluxed the Hoechst 33342 dye. Dye efflux from A431 SP cells was inhibited in the presence of 50  $\mu$ M verapamil, evidenced by decreased SP cells from 0.15 % to 0.02 % (data not shown). **(E)**: Flow cytometry analysis for SP was performed using ABCG2 (-) cells **(E1)** or ABCG2 (+) cells **(E2)**, showing that ABCG2 (-) cells contained 0.06 % of SP cells and 99.94 % of non-SP cells, while ABCG2 (+) cells did not contain SP cells.

**Fig. 4 (A1, A2)**: Flow cytometry analysis of cells from A431 xenograft showing a

distinctive population of integrin  $\beta 1$  (+) cells (12.69 %). **(B):** Flow cytometry analysis of cells from A431 xenograft demonstrating SP cells in Region 1 (R1) **(B1)** which effluxed the Hoechst 33342 dye. Dye efflux from A431 SP cells was inhibited in the presence of 500  $\mu$ M verapamil, evidenced by decreased SP cells from 3.25 % in **(B1)** to 0.24 % in **(B2)**. **(C):** Flow cytometry analysis for SP was using integrin  $\beta 1$  (+) cells **(C1)** or integrin  $\beta 1$  (-) cells **(C2)**, showing that integrin  $\beta 1$  (+) cells contained 0.29 % of SP cells and integrin  $\beta 1$  (-) cells contained 3.21 % of SP cells.

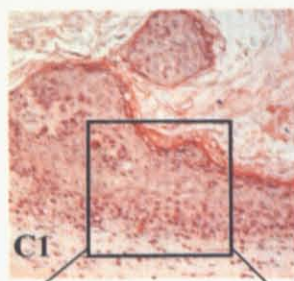
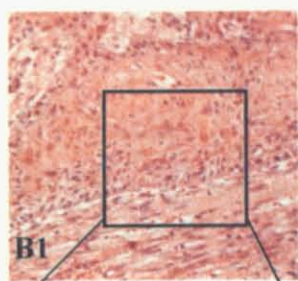
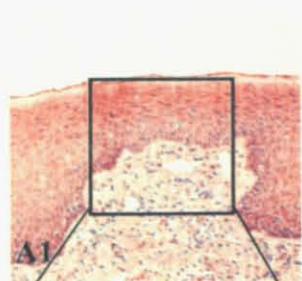
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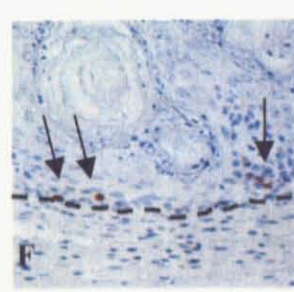
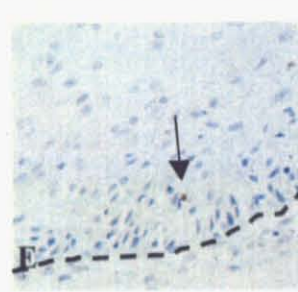
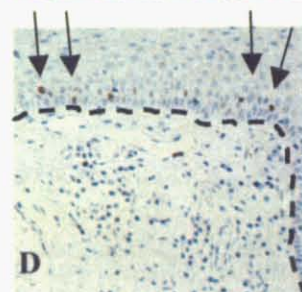
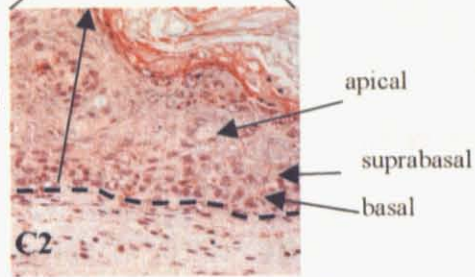
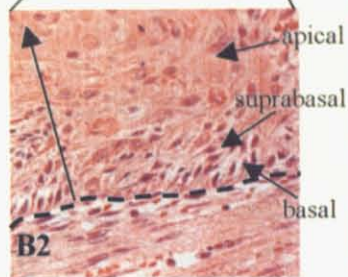
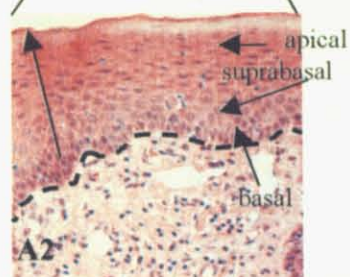
Normal epidermis

Squamous cell carcinoma

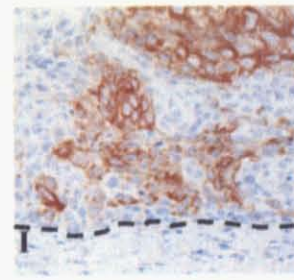
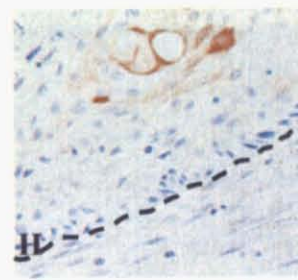
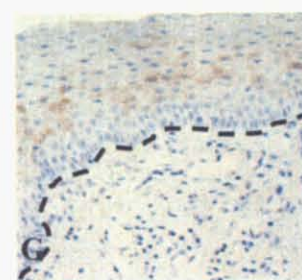
A431 xenograft



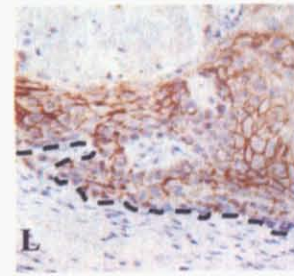
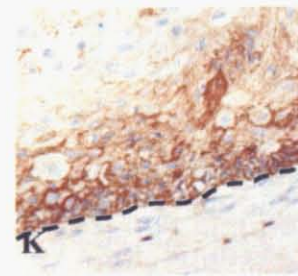
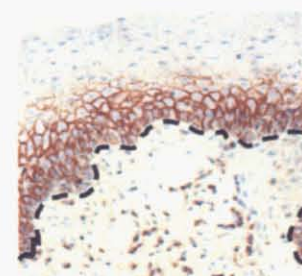
H & E



Ki-67



Cytokeratin 1



CD 44

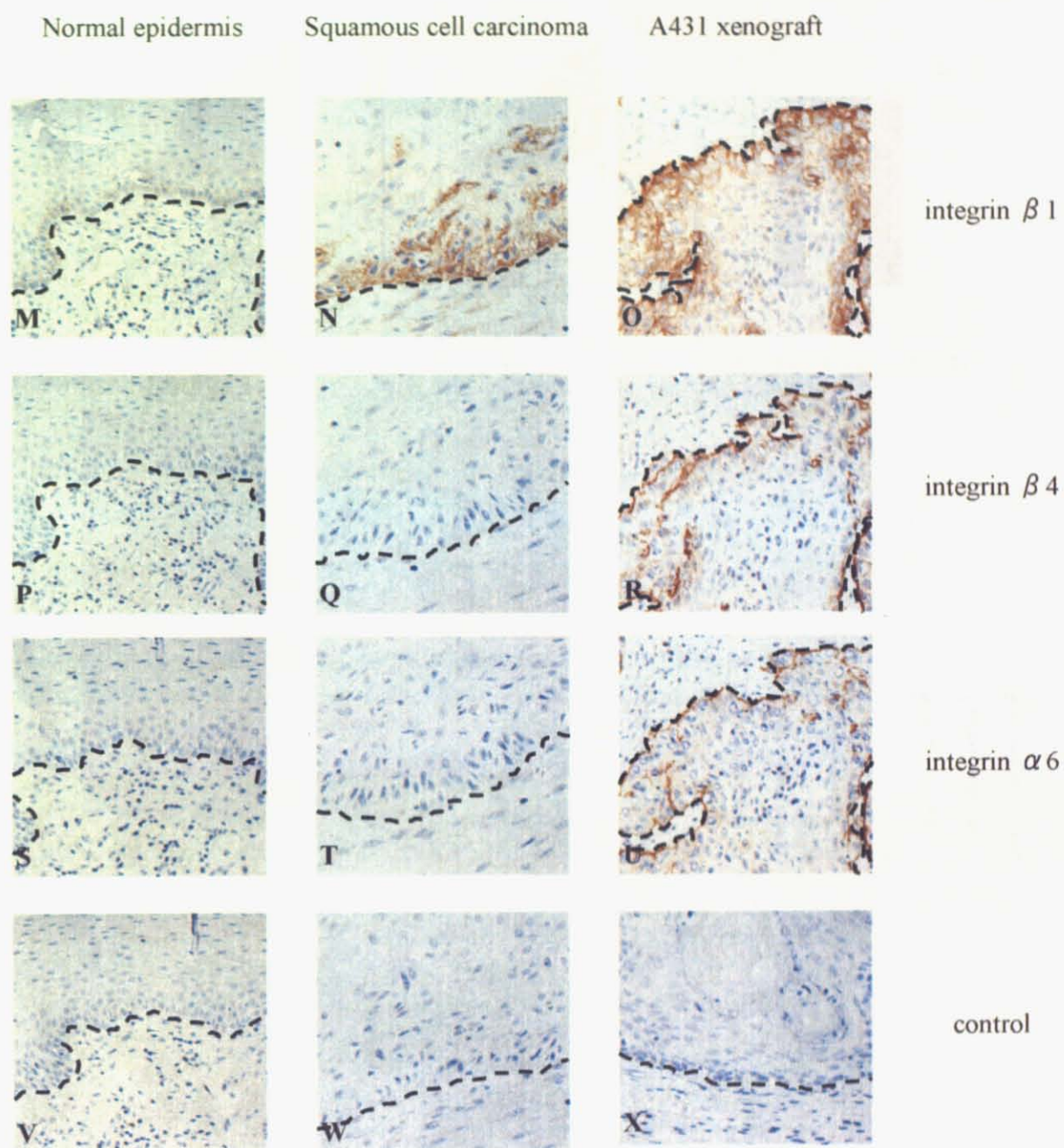


Fig. 1

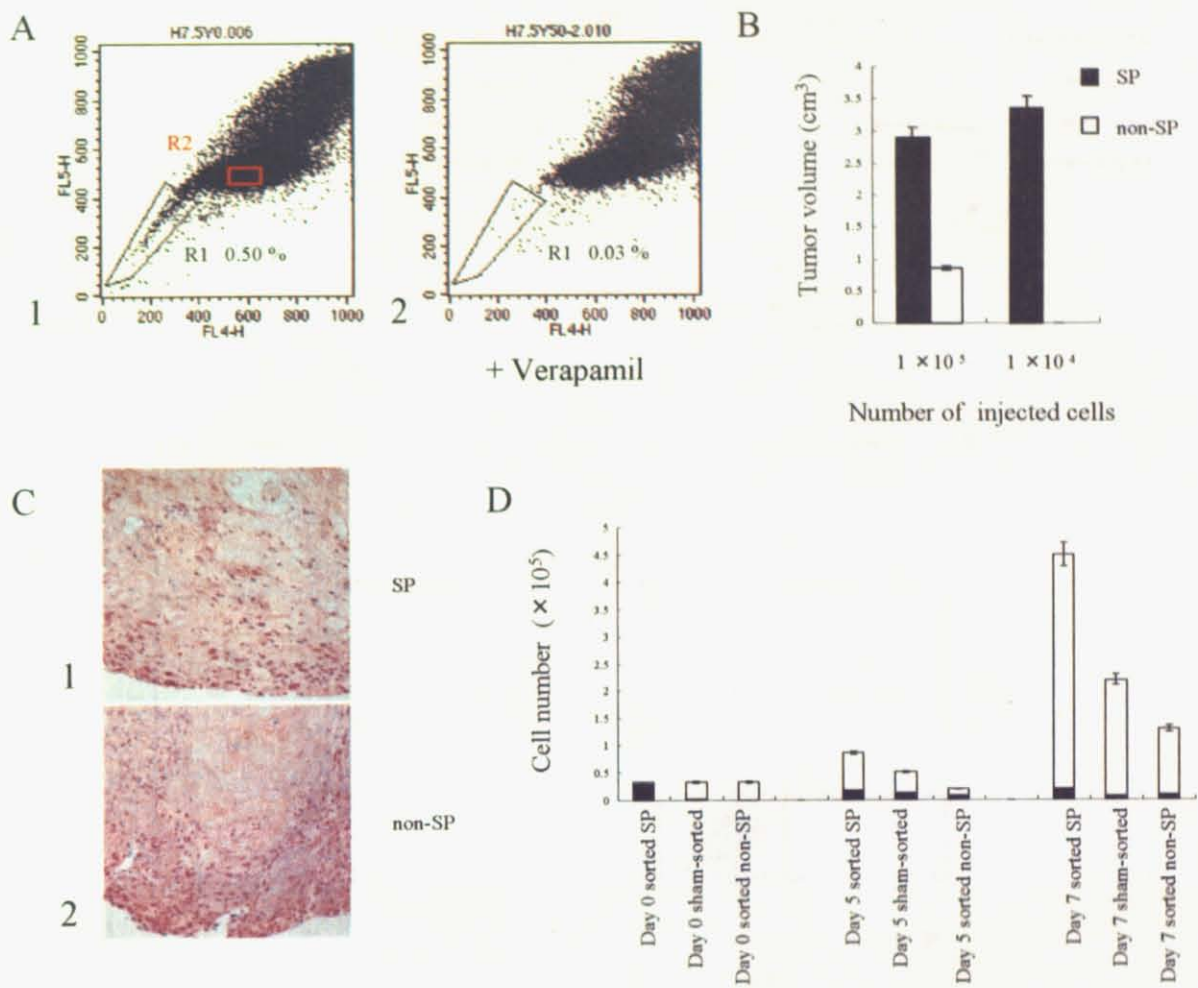


Fig. 2

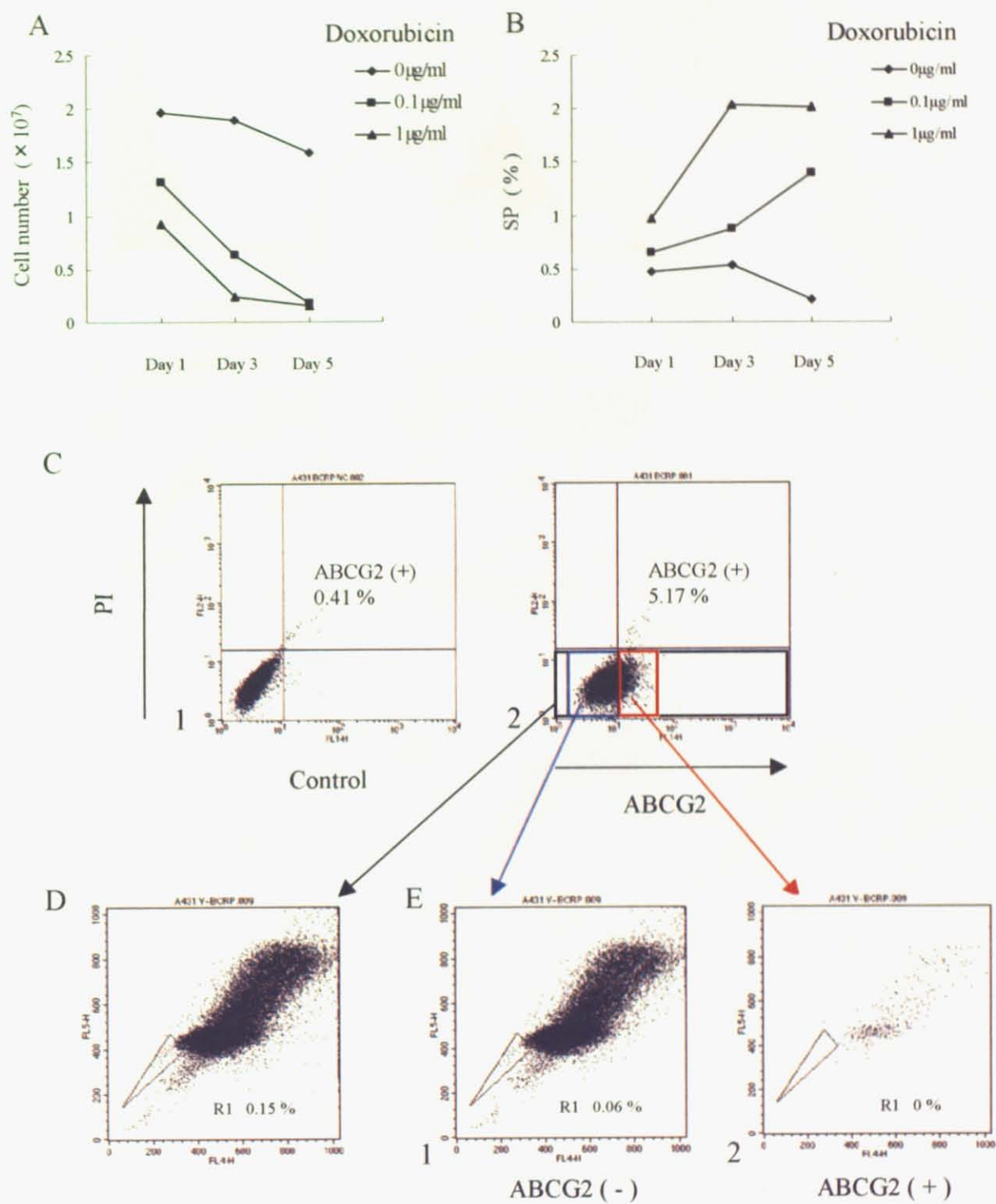


Fig. 3

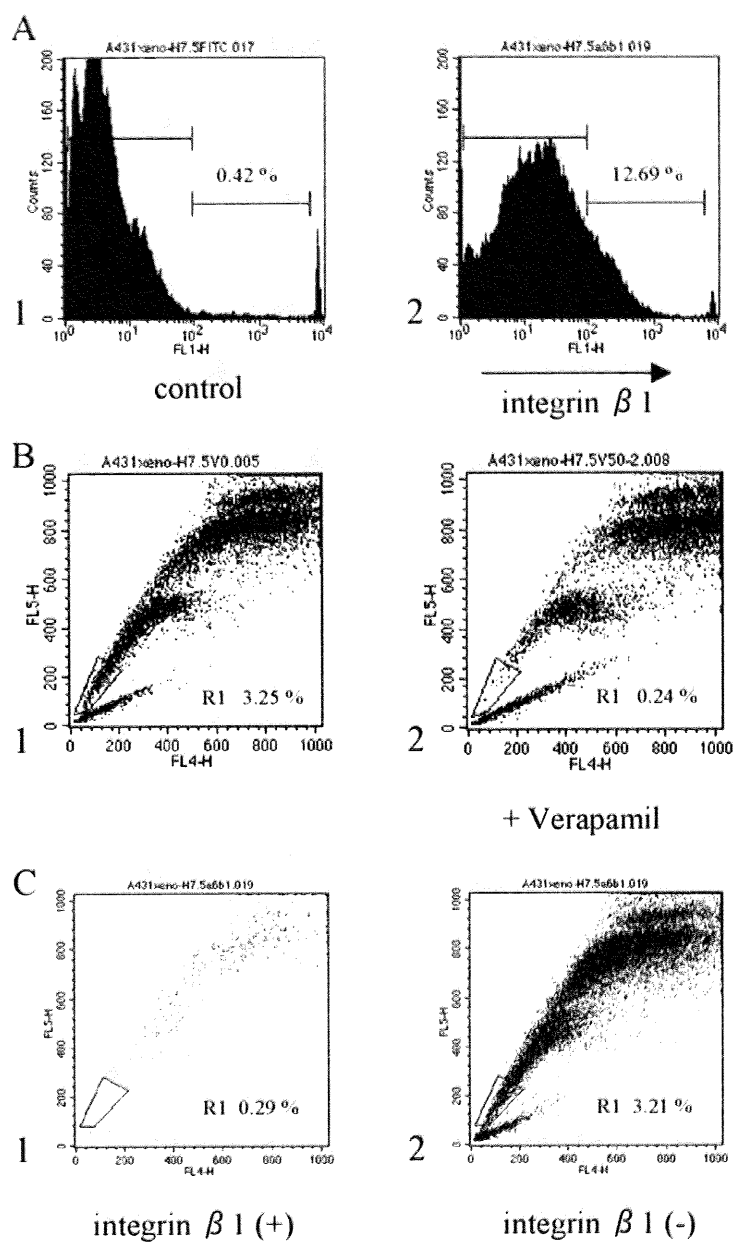


Fig. 4

**Table 1. Tumorigenicity of ABCG2 (-) cells and Sham-sorted Cells in SCID**

**Xenotransplant assay**

	Cell Number for Injection			
	$1 \times 10^3$	$1 \times 10^4$	$1 \times 10^5$	$1 \times 10^6$
ABCG2 (-) cells	0 / 3	0 / 3	0 / 3	1 / 1
Sham-sorted cells	0 / 3	0 / 3	0 / 3	1 / 1

**Table 2. Expression Properties of Integrins in Cultured A431 Cells and the Cells from A431 Xenograft**

	Cutured A431	A431 xenograft
		( % )
integrin $\alpha$ 2 ( CD49b )	99.6	45.2
integrin $\alpha$ 3 ( CD49c )	99.7	68.2
integrin $\alpha$ 6 ( CD49f )	99.8	25.7
integrin $\alpha$ V ( CD51 )	99.0	59.2
integrin $\beta$ 1 ( CD29 )	99.4	12.7
integrin $\beta$ 4 ( CD104 )	90.0	25.6