Biochemical and Biophysical Research Communications 373 (2008) 36–41

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Podoplanin, a novel marker of tumor-initiating cells in human squamous cell carcinoma A431

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ARTICLE INFO

Article history:
Received 20 May 2008
Available online 6 June 2008

Keywords:
Podoplanin
Squamous cell carcinoma
Tumor-initiating cells
Cancer stem cells
CD44
Sonic hedgehog

ABSTRACT

Squamous cell carcinoma (SCC) is a malignant tumor that shows morphologic and phenotypic similarities to normally differentiated squamous epithelium. Thus, it may be an ideal model for seeking a marker of tumor-initiating cells (TICs) based on their morphology. Using the human SCC cell line A431, we found that, as a paradigm of cancer stem cells: (1) podoplanin$^+$ cells generate both podoplanin$^+$ and podoplanin$^-$ cells; (2) podoplanin$^-$ cells rarely generate podoplanin$^+$ cells; (3) podoplanin$^+$ cells have higher colony formation efficiency and tumorigenicity than podoplanin$^-$ cells; (4) localization and morphology of podoplanin$^+$ cells in a xenografted tumor derived from podoplanin$^+$ cells are similar with those in human oral SCC tissue or normal epithelium. Furthermore, podoplanin$^+$ A431 cells share sonic hedgehog and CD44 expression with stem cells in normal squamous epithelium. Hence, we concluded that podoplanin is a novel marker to enrich TICs with stem-cell-like properties from SCC cell line A431.

In leukemia and some other solid cancers, only a rare subpopulation is clonogenic *in vitro* and *in vivo* [1,2]. To explain this phenomenon, a new hypothesis is proposed suggesting that only a small and phenotypically distinct subset of cells is responsible for generating and sustaining tumors and that this subset could be considered as cancer stem cells or tumor-initiating cells (TICs) [2–4].

Well-differentiated keratinizing squamous cell carcinoma (SCC) is a malignant tumor that is similar to normal stratified squamous epithelium with respect to its morphology and phenotype. Normal squamous epithelium consists of several layers differentiating from the basal layer, which contains stem cells, to the apical layers, which contain mature cells. On the other hand, the cells at the basal region of an SCC tumor nest have basal cell morphology and express CK5/14, the basal cell marker in normal squamous epithelium [5]. Furthermore, the cells at the center of the tumor nest show mature squamous morphology. Considering that the components of SCC are heterogeneous and that its histology and marker expression are similar to those of normal epithelium suggests a developmental hierarchy. Thus, based on the concept of cancer stem cells, the cells at the basal region of the SCC tumor nests could contain TICs with stem-cell-like properties. In fact, CD44, which is identified as a TIC marker of head and neck SCC, is expressed in the basal layer of cancer nests [5]. As it appears that SCC has a developmental hierarchy, it may be an ideal model for seeking a morphology-based TIC marker. To identify a candidate TIC marker of SCC that is useful both *in vitro* and *in vivo*, and considering that TICs might be expected to share some antigenic properties with normal tissue stem cells of the same organ [3,6–9], we screened known normal epithelial stem or progenitor cell markers [10,11] and basal cell markers [11,12] to search for an antigen that is expressed in the basal region of SCC. Furthermore, in order to use the same marker to obtain TICs from cultured cells, we investigated whether these putative TIC markers were also expressed in defined subpopulations of the SCC cell line A431 [13,14].

We defined stem-cell-like properties to mean the dual potential for differentiation and reproduction possessed by normal stem cells [15]. Normal stem cells divide asymmetrically to generate a stem cell and a progenitor, which maintains a heterogeneous cell population in the organ system it serves. A normal stem cell reproduces, giving rise to another stem cell, thus resulting in higher colony formation efficiency (CFE) than that of committed progenitors that are capable of further differentiation and proliferation but lack the ability to self-renew. In contrast, tumorigenicity and capability of recapitulating human SCC are by definition properties of TICs.

We identified podoplanin as a candidate TIC marker that could be useful both *in vitro* and *in vivo*, and tested whether podoplanin$^+$ A431 cells had stem-cell-like properties, tumorigenicity and capability of recapitulating human SCC. As podoplanin$^+$ A431 cells had these properties, we concluded that podoplanin is a marker of TIC in the SCC line A431.

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doi:10.1016/j.bbrc.2008.05.163

Materials and methods

Culture of A431 cell line. The human squamous cell carcinoma cell line A431 was obtained from RIKEN BioResource Center (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS, Sigma), and 1% penicillin and streptomycin (Sigma) and incubated at 37 °C in an atmosphere containing 5% CO_2.

Flow cytometry. Cultured A431 cells were counted and suspended in 100 μl of PBS/2% FBS per 10^6 cells. The A431 cell suspension was stained with anti-CD44 (unconjugated or phycoerythrin-conjugated, clone IM7, eBioscience, San Diego, CA) and anti-podoplanin (gp36, clone 18H5, Abcam, Cambridge, UK) antibodies. When unconjugated antibody was used, it was detected with appropriate secondary antibodies, depending on the experiment. Stained cells were washed and resuspended in 0.5 ml per 1×10^6 cells. Flow cytometry was performed using a FACSCalibur (Becton Dickinson (BD), Franklin Lakes, NJ) or a FACSAria (BD). Podoplanin$^+$ or podoplanin$^-$ cells were sorted and a small portion of them was reanalyzed to check for purity of the sorted population.

Immunohistochemistry. Immunohistochemistry of normal oral mucosa, oral SCC tumor subjects, and A431 xenografts was performed using paraffin-embedded sections to evaluate the expression of CD44 and podoplanin. Sections were deparaffinized and heated in the manufacturer's recommended unmasking solution at 95 °C for 20 min. The endogenous peroxidases were quenched with 0.3% H_2O_2 in PBS. For CD44 staining, sections were incubated with the primary antibody diluted in PBS containing 2% BSA and 0.1% NaN_3 for 1 h at room temperature, using anti-CD44 (clone DF1485, Novocastra Laboratories, Newcastle, UK). For podoplanin staining, sections were incubated overnight at 4 °C, using anti-podoplanin (clone D2-40, SIGNET, Dedham, MA). Following this incubation, sections were incubated with Envision$^{+®}$ system (DakoCytomation, Glostrup, Denmark) for 30 min at room temperature. Staining each sample without adding anti-human primary antibodies was performed as a negative control. Finally, samples were incubated with diaminobenzidine peroxidase substrate to give a brown stain and counterstained with hematoxylin, and mounted with cover-slips.

Single-cell colony formation assay. Sorted cells were placed into 24-well plates at 3.3×10^4 cells/well and cultured in the same conditions as unsorted A431. When they reached near confluence, the cells were trypsinized, washed twice with PBS and subcultured for propagation. The subcultured cells (passage 1) were collected and used for single-cell colony formation assay. Cells were suspended in DMEM/10% FBS and placed into 96-well plates at 0.5 cells in 100 μl/well. Thirty minutes after plating, each well was checked for the presence of a solitary cell. Four weeks after plating, the number of wells in which a colony had formed from the single cell was counted. The CFE was defined as the ratio of the number of colonies to the number of cells plated.

Cell transplantation into the severe combined immunodeficient (SCID) mice. Sorted cells were propagated *in vitro* and collected as described above. Varying numbers of sorted cells or unsorted A431 cells were suspended in a volume of 200 μl of DMEM/10% FBS and injected subcutaneously into 8–12-week-old female SCID mice (CLEA, Tokyo, Japan). The xenograft tumors were measured weekly, and at 6 weeks after transplantation, the xenograft was removed and processed for paraffin-embedded sections. The mice in which no tumor was detected were killed at 3 months after injection.

Real-time reverse transcriptase–polymerase chain reaction (RT–PCR). Sorted cells were pelleted, washed twice with PBS, resuspended in 1 ml of TRIzol (Invitrogen, Carlsbad, CA) and stored at −80 °C. Total RNA was purified by standard techniques from thawed samples, then cDNA was synthesized using the PrimeScript® RT reagent Kit (TaKaRa, Shiga, Japan), as directed by the manufacturer. RT–PCR was performed in a Smart Cycler® Systems (TaKaRa) using SYBR® Premix Ex Taq™ (TaKaRa) following the manufacturer's directions. The primers were shown in supplementary table 1.

Statistical analysis. Student's *t*-tests were performed. *P* values less than 0.05 were considered statistically significant.

Results

Identifying a candidate marker for TIC of SCC useful both in vivo and in vitro

Because TICs share expression of some markers with normal stem cells in the same organ [3,6–9], we assumed that TICs in SCC shared some marker expressions with normal stem cells in squamous epithelium. We investigated the expression of integrins β1, β4, α6, and of CD44 and podoplanin in normal oral mucosa, in oral SCC, in an A431 xenograft, and in cultured A431. Integrins β1, β4, α6, and CD44 are markers of normal epithelial stem or progenitor cells [10,11] and podoplanin is expressed in the basal layer of epithelium [12]. CD44 [5] and podoplanin [16] are also expressed in the basal layer of SCC tumor nests. Specific expression of integrins β1, β4, and α6 in the basal layer of normal oral mucosa was confirmed and their expression in oral SCC was limited to the basal region of cancer nests (data not shown). CD44 was highly expressed in the basal layer of normal mucosa and in the basal region of cancer nests. However, broad expression in cells with more differentiated morphology was also observed in both normal (Fig. 1A) and cancer (Fig. 1B) tissue. Although expression of integrins (data not shown) and CD44 (Fig. 1A and B) was limited in cells with immature morphology in normal mucosa and in cancer, almost all cultured A431 cells were positive for integrins (data not shown) and CD44 (Fig. 1D), indicating that these are not suitable markers to isolate rare subpopulations of TICs from A431 *in vitro*. In contrast, podoplanin was expressed specifically in the basal layer both in normal (Fig. 1E) and in cancer (Fig. 1F) tissues and a limited subpopulation (38.3 ± 13.0%, means ± SD) of cultured A431 was podoplanin$^+$ (Fig. 1H), indicating that podoplanin could be a candidate marker for TIC of SCC that could be useful both *in vivo* and *in vitro*. Xenograft tumors of A431 (Figs. 3D, 1C,G) were similar to human SCC (Figs. 3C, 1B,F), in their histology and expression of tested antigens, which indicates that A431 is an appropriate cell line capable of recapitulating human SCC.

Podoplanin$^+$ A431 cells have stem-cell-like properties in vitro

We tested whether podoplanin$^+$ A431 cells had the dual potential for differentiation and reproduction. The sorted podoplanin$^+$ cells were 97.7% podoplanin expressing (Fig. 2B). At day 5, this decreased to 21.6% (Fig. 2C), indicating that podoplanin$^+$ A431 cells generated both podoplanin$^+$ and podoplanin$^-$ cells. In contrast, we could not detect podoplanin$^+$ cells in sorted podoplanin$^-$ cells after five days' culture (Fig. 2F), nor after three passages (data not shown). These tendencies were also observed in clones of podoplanin$^+$ (day 28, Fig. 2D) or podoplanin$^-$ (day 28, Fig. 2G) cells. There was no significant difference in proliferation between podoplanin$^+$ and podoplanin$^-$ cells (Fig. 2H), while CFE of podoplanin$^+$ cells was significantly higher than that of podoplanin$^-$ cells (Fig. 2I). Hence, podoplanin$^+$ A431 cells have stem-cell-like properties *in vitro*.

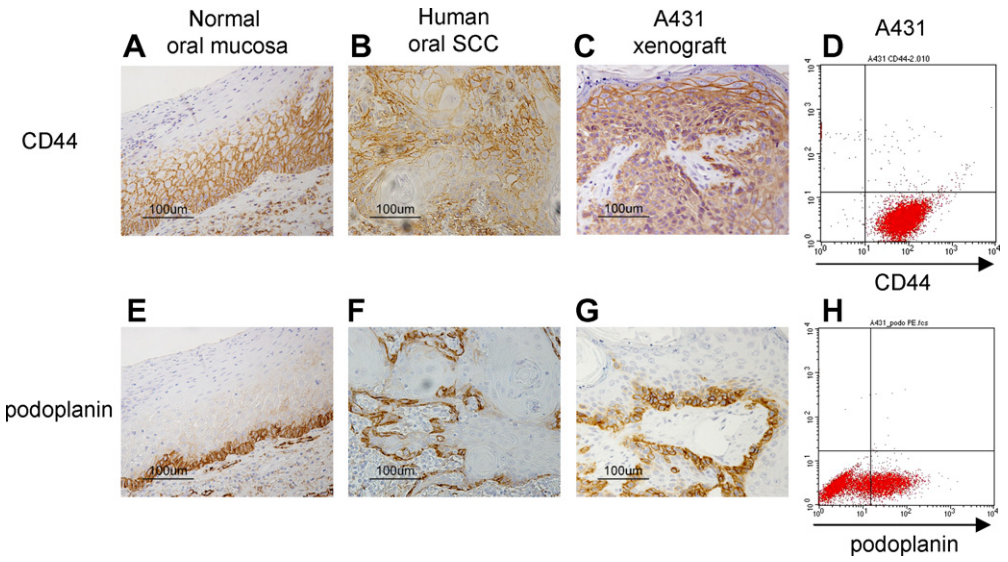

Fig. 1. Immunohistochemistry for CD44 and podoplanin in normal oral mucosa (A,E), human oral SCC (B,F) and xenograft tumors of A431 (C,G), and flow cytometric analysis of expression of these antigens in cultured A431 cells (D: CD44; H: podoplanin).

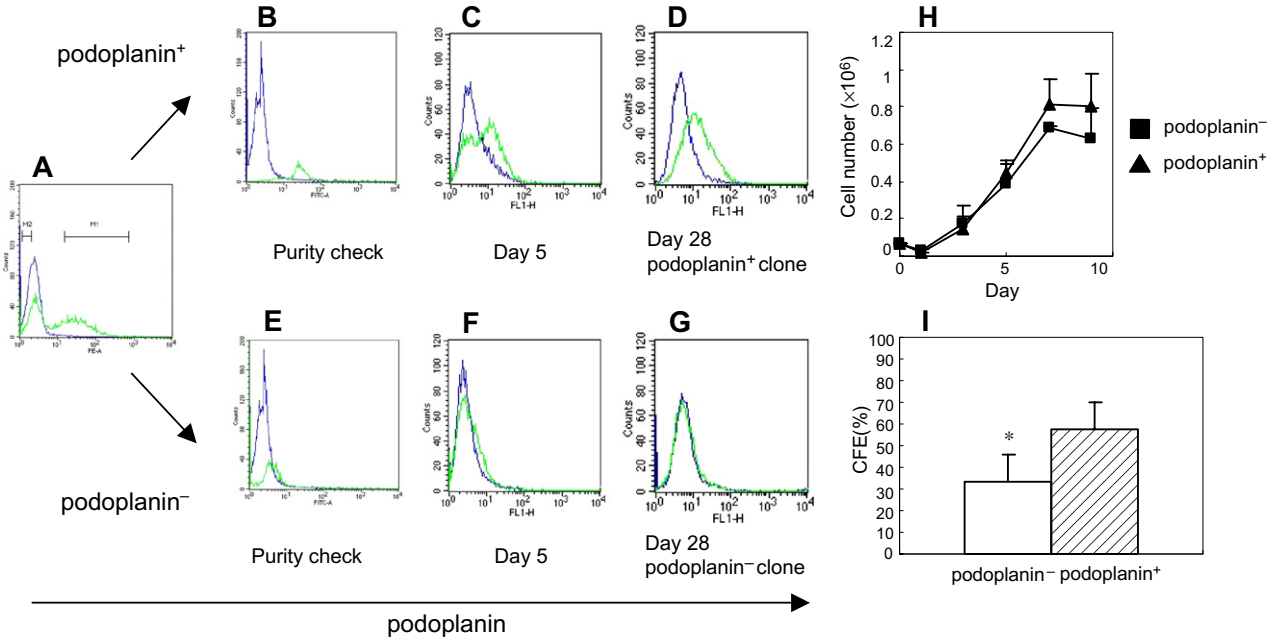

Fig. 2. Properties of podoplanin$^+$ and podoplanin$^-$ cells *in vitro*. Podoplanin$^+$ and podoplanin$^-$ cells were isolated by fluorescence activated cell sorting using the gate shown in (A). Sorted cells were reanalyzed to check purity (B,E) and propagated *in vitro*. At day 5, podoplanin expression was analyzed by flow cytometry (C,F). Colony formation assays were performed and podoplanin expression was analyzed by flow cytometry in colonies from podoplanin$^+$ cells (D) and from podoplanin$^-$ cells (G). Representative data are shown ($n = 8$ (podoplanin$^+$); $n = 15$ (podoplanin$^-$)). Sorted cells were seeded and counted at days 1, 3, 5, 7, and 9 (H) ($n = 3$). CFE was defined as the ratio of colony number to the number of plated cells (I) ($n = 3$). Bars represent SD ($^*P < 0.05$).

Podoplanin$^+$ A431 cells are tumorigenic and recapitulate human SCC

We investigated whether they also behaved in the stem-cell-like manner in animal models to initiate and sustain the growth of heterogeneous tumors. Therefore, we injected varying numbers of unsorted cells, sorted podoplanin$^-$ cells, or sorted podoplanin$^+$ cells into SCID mice and compared their tumorigenicity and histology. At four weeks after injection, 10^4 podoplanin$^+$ cells were sufficient to generate tumors, while 10^5 unsorted cells or 10^6 podoplanin$^-$ cells were required for tumor generation (Fig. 3A). There was no significant difference in the doubling time of tumor between unsorted cells and podoplanin$^+$ cells, judged by the inclination of the graph of tumor volume (Fig. 3B). The volumes of tumors arising from 10^4 podoplanin$^+$ cells were greater than those generated from 10^4 unsorted cells (Fig. 3B) when no tumors were generated by 10^4 podoplanin$^-$ cells. The latency of 10^4 podoplanin$^+$ cells (2 weeks; Fig. 3B) was shorter than that of 10^4 unsorted cells (5 weeks; Fig. 3B) or 10^4 podoplanin$^-$ cells (10 weeks; data not shown). The shortest latencies of podoplanin$^+$ cells were also observed when 10^6, 10^5, or 10^3 cells were injected (data not shown). These data indicate that podoplanin$^+$ cells are enriched for tumorigenic cells. Moreover, tumors from podoplanin$^+$ cells (Fig. 3E and

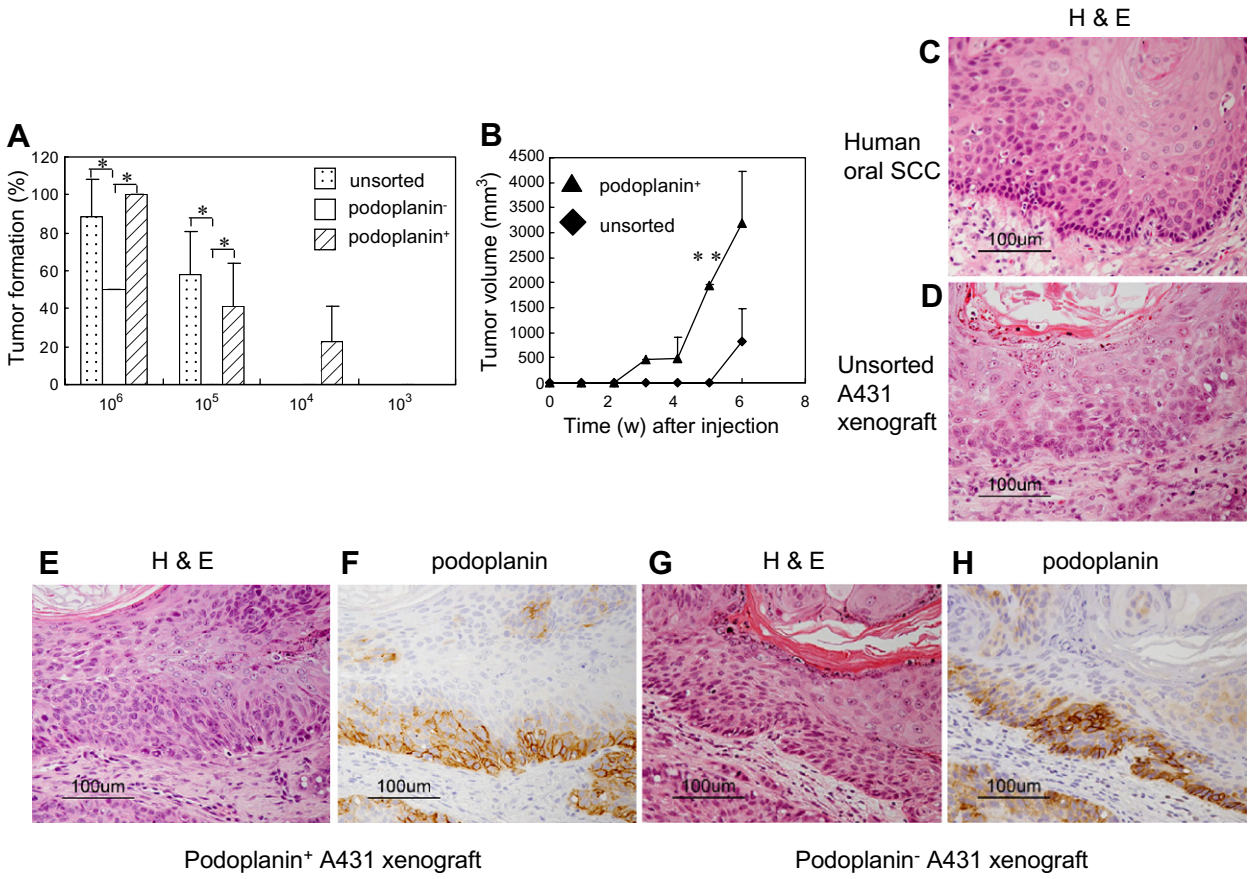

Fig. 3. Tumor-initiating potential of podoplanin$^+$ A431 cells in SCID mice. Varying numbers of sorted and propagated cells or unsorted cells were injected and data represent the percentage of mice with tumor formation at 4 weeks after injection (A, $n = 6–11$). The volume of tumors generated from 10^4 podoplanin$^+$ cells or unsorted cells are shown (B: $n = 4$ (podoplanin$^+$); $n = 2$ (unsorted)). Bars represent SD ($^*P < 0.05$ and $^{**}P < 0.01$, respectively). No tumors were generated by 10^4 podoplanin$^-$ cells by six weeks after injection. Hematoxylin and eosin (H&E) staining of human oral SCC (C), xenograft tumors from unsorted A431 cells (D), from podoplanin$^+$ (E) cells, or from podoplanin$^-$ (G) cells. Immunohistochemistry for podoplanin in xenograft tumors from podoplanin$^+$ (F) or podoplanin$^-$ (H) A431 cells.

F) recapitulated both well-differentiated histology and podoplanin expression, showing similar features as tumors arising from unsorted A431 cells and human SCC (Figs. 3D,C, 1G,F). These data suggest that podoplanin$^+$ cells are enriched for TICs. However, podoplanin$^-$ A431 cells also had the ability to form tumors recapitulating both well-differentiated histology and podoplanin expression of human SCC (Fig. 3A,G,H). This could be explained as that TICs were also included at a lower density within podoplanin$^-$ A431 cells. Alternatively, podoplanin expression may be induced within a specific microenvironment under *in vivo* condition, although we could not rule out the possibility that it was due to the contamination of small number of podoplanin$^+$ cells.

Podoplanin expression correlates with the expression of SHH or CD44

We investigated the expression of Sonic hedgehog (SHH) [17], CD44 [5,6,13], BMI1 [5] and ABCG2 [18], prominin 1 (CD133: PROM1) [4,9,14,19–22], genes that have been reported to be expressed in normal stem cells or TICs. We found that mRNA for SHH was expressed specifically in sorted podoplanin$^+$ A431 cells (Fig. 4B). Expression of mRNA for CD44 (Fig. 4C), which has been demonstrated to be a marker of TICs in head and neck SCC tissues from human specimens [5], correlated with podoplanin expression in A431 cells. There was no significant difference in the expression of BMI1 (Fig. 4D), ABCG2 (Fig. 4E), and prominin 1 (Fig. 4F) between unsorted cells, sorted podoplanin$^-$ cells, and sorted podoplanin$^+$ cells.

Discussion

We identified podoplanin as a new marker for TIC of SCC. Podoplanin$^+$ A431 cells possessed stem-cell-like properties *in vitro* because they had the ability to repopulate and to generate a heterogeneous cancer cell population (Fig. 2B–D). Furthermore, we confirmed that podoplanin$^+$ A431 cells had high tumor-initiating potential (Fig. 3A and B) and were capable of recapitulating human SCC (Fig. 3E and F), which is sufficient to consider podoplanin as a TIC marker [6].

We found SHH to be exclusively expressed in podoplanin$^+$ A431 cells (Fig. 4B). This indicates not only the similarity of TICs in SCC to SHH-expressing pancreatic TICs [17], but also the similarity of TICs in SCC to SHH-expressing normal stem cells in squamous epithelium [23]. Involvement of SHH signaling in the anchorage-independent growth of SCC has been shown using human SCC cell lines including A431 [24]. In this study, we showed that cultured cells were heterogeneous for SHH expression. Podoplanin may serve as a marker to isolate SHH-expressing cells and may allow more efficient investigation of SHH signaling in SCC.

The role of podoplanin in tumor initiation remains elusive. Its involvement in tumor metastasis, however, has been demonstrated to be due to its platelet aggregation-inducing activity leading to pulmonary retention of CHO cells that overexpress podoplanin [25]. Podoplanin also contributes to tumor invasion by binding ERM proteins to activate RhoA resulting in epithelial-mesenchymal transition [26]. Although podoplanin$^+$ TICs in SCC

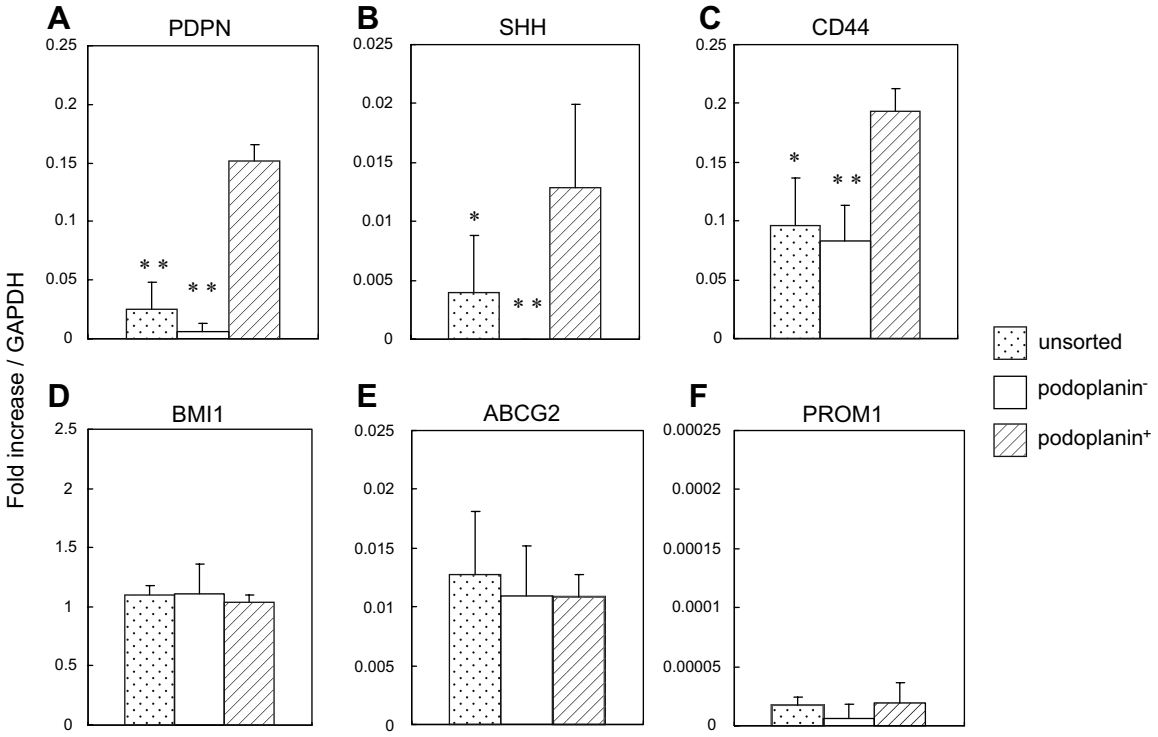

Fig. 4. Expression of podoplanin/PDPN (A), Sonic hedgehog/SHH (B), CD44 (C), BMI1 (D), ABCG2 (E), and prominin 1/PROM1 (F) in unsorted cells, podoplanin$^-$ cells, and podoplanin$^+$ cells. RNA from sorted cells was reverse transcribed and real-time quantitative RT-PCR was performed. The relative expression of genes is plotted as a ratio of GAPDH gene expression. Data represent the average value from three independent experiments in duplicate. Bars represent SD ($^*P < 0.05$ and $^{**}P < 0.01$, respectively).

may use these mechanisms to initiate and sustain tumor growth, they may also proliferate rapidly because of activation of the SHH signaling pathway. In addition to these intrinsic mechanisms, there also seems to be a contribution of the microenvironment to the ability of TICs to generate tumors, because the increased tumorigenicity of podoplanin$^+$ cells over podoplanin$^-$ cells *in vivo* (Fig. 3A) was greater than the increase in CFE *in vitro* (Fig. 2I). Histologically, podoplanin$^+$ cells were specifically located at the basal region of SCC tumor nests, which was close to the surrounding stromal cells. Considering their localization, we speculate that TICs may be regulated by stromal cells, which is comparable to the regulation of stem cells by their environmental niche [15].

We also showed a correlation between podoplanin and CD44 expression in A431 cells (Fig. 4C), congruent with the 1.4- to 1.5-fold enriched podoplanin $^+$ cells in CD44 strongly expressing A431 cells detected by flow cytometry (data not shown). Because CD44 is a marker of TICs in SCC tissue [5], podoplanin$^+$ A431 cells may be also considered as TICs. Podoplanin$^+$ A431 cells may serve as a good source of cells that resemble TICs in tissue.

Because TICs are considered to resist conventional therapies [27,28] and to be responsible for relapse [27], targeting TICs is proposed as an effective approach to cancer therapy. As podoplanin is a surface marker, specific antibody therapy for podoplanin may be available to attack TICs.

Acknowledgments

We thank Hiroko Hashimoto and Mai Okumoto for technical support and Dr. Syuichi Mitsunaga, Takashi Ito and Ayuko Hoshino for their helpful advice. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare; a Grant for Scientific Research Expenses for Health Labor and Welfare Programs; the Foundation for the Promotion of Cancer Research, the 3rd-Term Comprehensive 10-Year Strategy for Cancer Control.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.163.

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