PC3 Human Prostate Carcinoma Cell Holoclones Contain Self-renewing Tumor-Initiating Cells

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Abstract

Primary keratinocytes exhibit three typical clonal morphologies represented by holoclones, meroclones, and paraclones, with holoclones containing self-renewing stem cells, and meroclones and paraclones containing more mature and differentiated cells. Interestingly, long-term–cultured human epithelial cancer cells in clonal cultures also form holoclones, meroclones, and paraclones, and tumor cell holoclones have been hypothesized to harbor stem-like cells or cancer stem cells. However, the key question of whether tumor cell holoclones genuinely contain tumor-initiating cells has not been directly addressed. Here, using PC3 human prostate carcinoma cells as a model, we provide direct experimental evidence that tumor cell holoclones contain stem-like cells that can initiate serially transplantable tumors. Importantly, holoclones derived from either cultured PC3 cells or holoclone-initiated tumors can be serially passaged and regenerate all three types of clones. In contrast, meroclones and paraclones cannot be continuously propagated and fail to initiate tumor development. Phenotypic characterizations reveal high levels of CD44, $\alpha_2\beta_1$ integrin, and β-catenin expression in holoclones, whereas meroclones and paraclones show markedly reduced expression of these stem cell markers. The present results have important implications in understanding morphologic heterogeneities and tumorigenic hierarchies in human epithelial cancer cells. [Cancer Res 2008;68(6):1820–5]

Introduction

Every normal tissue or organ comprises multiple resident cell types that are heterogeneous with respect to their morphologies, functions, and gene and protein expression patterns. This cellular heterogeneity has been thought to reflect mostly the developmental and maturation stages of various normal stem and progenitor cells (1). Pioneering work by Barrandon and Green more than 20 years ago (2) showed that when primary human keratinocytes were put in culture, their abilities to establish a clone were related to the heterogeneity in cell size—only cells ≤11 μm in diameter could form a clone whereas cells ≥12 μm were irreversibly committed to terminal differentiation. Their subsequent work (3) in clonal cultures revealed three distinct types of clones with profoundly different proliferative capacity. The holoclone contains tightly packed small cells and has the greatest replicative capacity, and <5% of the colonies formed by the cells of a holoclone abort and terminally differentiate. In contrast, the paraclone is a loosely packed clone of large cells with a short replicative life span—after <15 cell generations, paraclones uniformly abort and terminally differentiate. The third type of clone, the meroclone, contains a mixture of cells of different proliferative potential and is a transitional stage between the holoclone and the paraclone (3). Recent work reveals that keratinocyte holoclones contain self-renewing stem cells and that the ability to form a holoclone is an intrinsic property of the adult stem cells of the hair follicle (4).

Tumor development to a certain degree resembles and has been compared as "caricatures" of normal tissue histogenesis and organogenesis (5). Indeed, most human tumors are heterogeneous in their cellular composition (6–8). Although many posit that tumor cell heterogeneity is of a genetic basis associated with inherent high genomic instability in tumor cells, the heterogeneous cellular composition in tumors has also been hypothesized, early on, to be the consequence of abnormal tumor stem cell differentiation (9). This latter postulate, called "cancer stem cell (CSC) hypothesis" was recently revived (10) mainly due to progress made on studies of normal tissue stem cells. The CSC hypothesis has two central tenets—tumors are derived from transformation of normal stem cells or their progeny (i.e., progenitor or even differentiated cells) and every tumor contains a small population of stem-like cells that possess a unique ability to drive tumor formation and maintain tumor homeostasis (10). In support of the first tenet, both chronic myelogenous leukemia (11) and acute myelogenous leukemia (12) seem to have arisen from the committed progenitor cells that have acquired self-renewing capabilities. In support of the second tenet, stem-like cells or CSCs that can initiate serially transplantable tumors in mice recapitulating the heterogeneous nature of patient tumors have been reported not only in leukemia (11–14) but also in solid tumors, including breast cancer (15), glioma (16), colon cancer (17–19), head and neck squamous cell carcinoma (20), and pancreatic cancer (21). Most of these CSCs have been identified using surface markers that identify their corresponding normal tissue stem and progenitor cells, thus reinforcing the resemblance of tumor development to normal organogenesis. Of great interest, stem-like cells have also been discovered in some cultured cancer cell lines, including glioma (22, 23), ovarian (24, 25), liver (26), and prostate (27–29) cancer cells.

Similar to primary keratinocytes, long-term–cultured squamous cell carcinoma (30) and many other epithelial cancer cells (30, 31) can also form different types of clones in culture. Only a small percentage of cells are endowed with the ability to establish holoclones, whereas the majority forms paraclones and meroclones (30, 31). Like keratinocyte holoclones, cancer cell holoclones have been hypothesized to contain self-renewing stem-like cells

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although direct evidence supporting this hypothesis is still lacking. In this study, we used PC3 cells, an androgen receptor–negative, undifferentiated prostate cancer cell line, as the model system to directly test this hypothesis.

Materials and Methods

Cells, animals, and reagents. PC3 cells were obtained from the American Type Culture Collection and cultured in RPMI containing 7% of heat-inactivated fetal bovine serum (FBS). Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were initially purchased from The Jackson Laboratory, and the breeding colonies were established in our animal facility (27–29) and maintained in standard conditions according to the institutional guidelines. Most chemicals and reagents were from Sigma unless otherwise specified.

Establishment of PC3-GFP cells. Basic retroviral and lentiviral procedures have been previously described (32, 33). Briefly, 293FT packaging cells were transfected with pLL3.7-GFP lentiviral vector, together with the packaging plasmids, using Fugene 6. The virus-containing medium was collected 48 to 72 h later and passed through a 0.45-μm filter to remove debris. PC3 cells were then infected with virus at a multiplicity of infection of 50 and essentially 100% cells were infected.

Single-cell cloning by limiting dilution. PC3-GFP cells (~70% confluent) were harvested in trypsin (0.25%)/EDTA and resuspended in fresh medium to generate a single-cell suspension with a density of ~10 cells/mL. Then, 100 μL single-cell suspension was dispensed into each well in a 96-well culture plate. After plating, each well was checked under both an Olympus BX50 fluorescence microscope and a phase-contrast microscope. Wells containing only a single cell were marked, and wells with no cells or with more than one cell were excluded. These single-cell wells were checked daily and maintained in RPMI-7% FBS. When the colony grew confluent, they were transferred to six-well dishes. Clones in six-well dishes were maintained until nearly confluent and then some of them were frozen and some were replated into T-75 flask or used for tumor experiment.

Senescence-associated β-galactosidase staining. Basic procedures were previously described (32).

Subcutaneous tumor cell implantation and purification of tumor cells from xenograft tumors. Basic procedures were previously described (27–29). For tumor development assays, cells derived from holoclones, meroclones, or paraclones were injected s.c. in 50% Matrigel into the flanks of NOD/SCID mice. To purify tumor cells, PC3 xenograft tumors were aseptically dissected out from animals and minced into ~1-mm^3 pieces in RPMI-7% FBS. After rinsing in the same medium (2×), tumor tissues were incubated with 1× Accumax (1,200–2,000 units/mL proteolytic activity containing collagenase and DNase; Innovative Cell Technologies, Inc.) at 20 mL/1 g tissue for ~30 min at room temperature under rotating conditions. Single-cell suspension was obtained by filtering the supernatant through a 40-μm cell strainer, and cell suspension was then gently loaded onto a layer of Histopaque-1077 gradient (1×10^6–3×10^6 cells/mL HistoPaque in a total of 3-mL volume) and then centrifuged at 400 × *g* for 30 min at room temperature. RBC, dead cells, and debris were eliminated from the bottom of the tube and live nucleated cells were collected at the interface. Then, the single-cell suspension was used for tumorigenesis or serial transplantation experiments.

Immunophenotypic characterizations of PC3 cell clones. Bulk-cultured PC3 or PC3-GFP cells, or clone-derived cultures were plated on glass coverslips and used in immunofluorescence staining for CD44, $\alpha_2\beta_1$ integrin, and β-catenin using monoclonal antibodies and protocols described previously (27–29).

Results and Discussion

Prostate cancer cells in clonal cultures exhibit distinct clonal morphologies. Our laboratory has been studying normal human prostate epithelial and prostate cancer stem and progenitor cells (27–29, 31–33). Our recent work has shown the existence of stem-like cells in cultured as well as xenograft-derived prostate cancer cells (27–29, 31). Cultured epithelial cancer cells, when

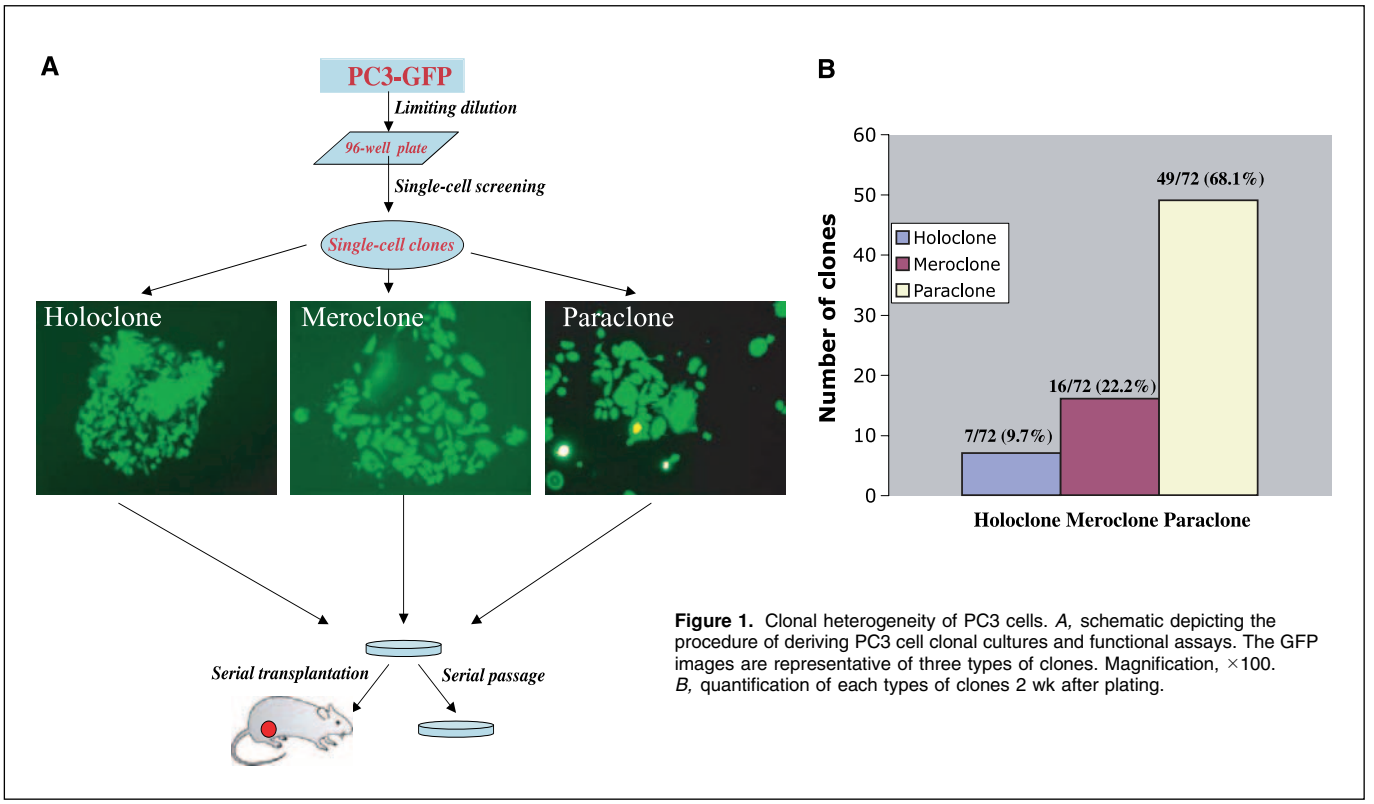

Figure 1. Clonal heterogeneity of PC3 cells. *A,* schematic depicting the procedure of deriving PC3 cell clonal cultures and functional assays. The GFP images are representative of three types of clones. Magnification, ×100. *B,* quantification of each types of clones 2 wk after plating.

A

Clone type	Survival	Aborted
Holoclone	6/7 (85.7%)	1/7 (14.3%)
Meroclone	11/16 (68.8%)	5/16 (31.2%)
Paraclone	1/49 (2.0%)	48/49 (98.0%)

B

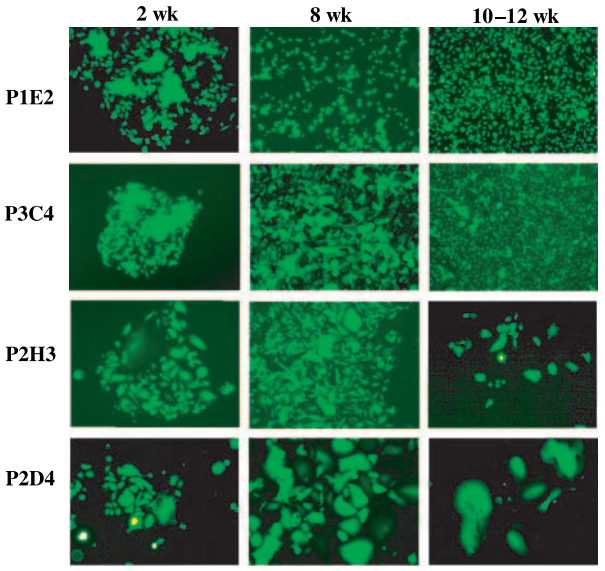

Figure 2. Developmental fate of three types of clones during the 12-wk follow-up period. *A,* a table showing the "survival" and "abort" percentages of three types of clones 8 wk after initial plating. *B,* representative GFP images of two holoclones (P1E2 and P3C4), one meroclone (P2H3), and one paraclone (P2D4). Original magnifications, ×200.

plated at clonal densities, have been shown to behave like primary keratinocytes and form colonies of distinct morphologies (30, 31). Indeed, when PC3 (Supplementary Fig. S1*A*) and Du145 (Supplementary Fig. S1*B*) prostate cancer cells were cultured at clonal densities, in ~2 weeks clones resembling keratinocyte holoclones, meroclones, and paraclones could be easily identified. Like keratinocyte clones (2–4), prostate cancer cell holoclones consisted of tightly packed, small cells and paraclones of larger and fewer cells, whereas the meroclones were made of cells of intermediate sizes and numbers (Supplementary Fig. S1). Importantly, although cells in prostate cancer cell holoclones were homogenously small and senescence-associated β-galactosidase (SA-βgal) negative, most cells in paraclones were flat and large and SA-βgal positive (Supplementary Fig. S1), suggesting that the latter cells were mostly senescent and nonproliferative. Many cells in meroclones were also SA-βgal positive (Supplementary Fig. S1*A*). These results reveal that prostate cancer cells are heterogeneous in establishing different types of clones.

Different PC3 cell clones possess distinct proliferative and self-renewing capacities. We previously showed that Du145 cell holoclones could be serially passaged and sustain long-term expansion in culture (31). In this study, we focused on PC3 cells, which lack androgen receptor expression, are completely undifferentiated, are highly tumorigenic and metastatic, and have been considered the most malignant prostate cancer cell line (27–29). We plated PC3-GFP cells at ≤1 cell per well in 96-well plates, and 2 hours after plating (to prevent cell division) we marked and selected those wells that contained only 1 viable cell (Fig. 1*A*). Among the three 96-well plates studied, 72 wells satisfied our selection criteria (see Materials and Methods) and were followed up on the cell development at the clonal level (Fig. 1*B*). In 2 weeks, 7 clones (~10%) developed into typical holoclones and 49 cells (68%) formed paraclones, whereas 16 clones (~22%) were meroclones (Figs. 1*A* and *B* and 2). We then painstakingly followed the development of each individual clone *in vitro* through serial passaging and, for several clones, we also carried out serial tumor transplantation experiments (Fig. 1*A*; see below). As shown in Fig. 2*B*, the distinct morphologies of three types of PC3 clones were obvious at 2 weeks after single-cell plating. By 8 weeks, although most holoclones proliferated robustly and only 1 holoclone aborted, most (i.e., 98%) of the paraclones, on the other hand, had aborted and thus been terminated (Fig. 2*A* and *B*). Meroclones

Table 1. Tumorigenicity and serial transplantability of PC3-GFP cell clones

Clone	Cell	Tumor incidence*	Mean tumor weight (g)†	Termination (d)‡
PC3	100	1/8 (13%)	ND	90 (33)
	1,000	5/8 (63%)	ND	75 (33)
	10,000	8/8 (100%)	ND	60 (18)
	100,000	7/8 (88%)	ND	45 (18)
PC3-GFP	10,000	7/8 (88%)	1.11	65 (24)
	100,000	6/8 (75%)	1.37	55 (11)
P3C4 (holoclone)				
	10,000	8/8 (100%)	0.37	70 (24)
2°	1,000	8/10 (80%)	0.39	69 (26)
3°	1,000	9/10 (90%)	0.51	47 (26)
P1E2 (holoclone)	1,000	8/10 (80%)	0.21	75 (54)
	10,000	9/10 (90%)	1.41	75 (47)
2°	1,000	5/6 (83%)	0.22	55 (35)
3°	1,000	7/8 (88%)	0.19	49 (29)
P1H8 (holoclone)	1,000	8/10 (80%)	0.27	69 (36)
	10,000	10/10 (100%)	1.39	69 (28)
2°	1,000	6/6 (100%)	1.06	71 (39)
P2A12 (meroclone)	10,000	0/10	0	187
	100,000	0/2	0	187
P2H3 (meroclone)	10,000	0/9	0	121
	100,000	0/2	0	121
P2D4 (paraclone)	1,000	0/1	0	63

NOTE: PC3 or PC3-GFP cells, or PC3-GFP cell clones at the indicated numbers were injected in 50% Matrigel into the flanks of NOD/SCID mice. Secondary (2°) and tertiary (3°) serial transplantation experiments were performed as described in the text.
Abbreviation: ND, not determined.
*The number of tumors developed/number of injections.
† Mean tumor weight in grams.
‡ Time in days when animals were terminated and the tumor was harvested. The numbers in parentheses are latency (i.e., when tumors were first detected after implantation).

showed intermediate behavior: By 8 weeks, 5 of the 16 clones (31%) were abolished and could not be further propagated (Fig. 2*A*). By 10 to 12 weeks after initial plating, 6 of the 7 holoclone-derived PC3 cells were still robustly proliferating whereas the remainder 1 paraclone and the rest of the meroclones all aborted (Fig. 2*B, right panels*). In all "aborted" clones, cells were generally big and flat (e.g., Fig. 2*B, bottom middle* and *right panels*) and SA-βgal positive (not shown). All 6 holoclone-derived PC3 cells could be continuously propagated for >6 months.

When the P1E2 holoclone-derived cells were replated at clonal densities, they were able to regenerate the full spectrum of clonal heterogeneities within ~1 week (Supplementary Fig. S2*A*).

PC3 holoclones, but not paraclones or meroclones, contain stem-like cancer cells that could initiate tumor development and sustain serial tumor transplantation. The above serial cell passaging and replating experiments show that the PC3 holoclones contain self-renewing cancer cells or CSCs that can sustain long-term propagation in culture. The gold standard in measuring CSC activities is that the candidate cell populations must be able to initiate serially transplantable tumor development (31, 34). Therefore, we determined and compared the tumor-initiating capacities of PC3 holoclones, meroclones, and paraclones. As shown in Table 1, both PC3 and PC3-GFP cells initiated cell number–dependent tumor development in NOD/SCID mice. As few as 100 PC3 cells initiated tumor development in one of the eight injections and at ≥10,000 cells most of the injections of either PC3 or PC3-GFP cells developed tumors (Table 1). Three holoclones (i.e., P3C4, P1E2, and P1H8), harvested around 6 to 8 weeks after plating, when injected at 1,000 or 10,000 cells, all initiated 80% to 100% tumor development in NOD/SCID mice in 47 to 75 days (Table 1; Fig. 3*A*). In sharp contrast, two meroclones (i.e., P2A12 and P2H3) did not develop any tumors in 5 to 6 months even with 10,000 or 100,000 cells (Table 1). Most paraclones became senescent very early on, and these senescent cells were very difficult to harvest. The only paraclone of which we managed to harvest 1,000 cells did not initiate tumor development (Table 1).

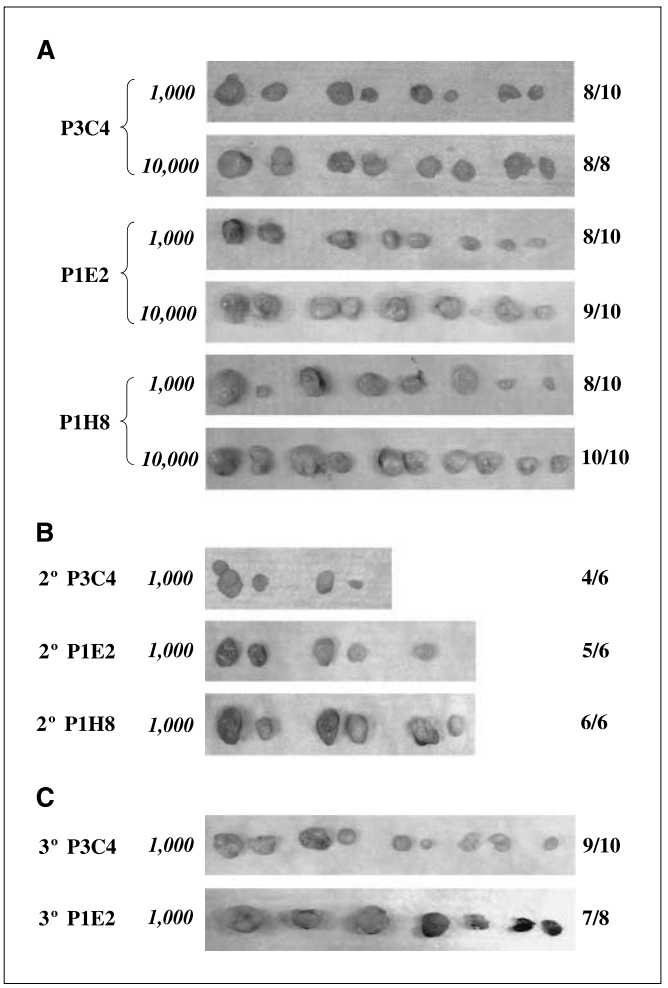

Figure 3. Serial tumor transplantation of three holoclone-derived PC3 cells. *A* to *C,* tumor images of the first, secondary, and tertiary, respectively, tumor transplants. There are no statistical differences in tumor rates among each clone ($P > 0.05$; Fisher's test).

Importantly, when the three holoclone-derived tumors were harvested and PC3-GFP cells were purified and used in secondary tumor transplantation experiments, 1,000 cells of each clone regenerated tumors in 70% to 100% of the injections (Table 1; Fig. 3*B*). When tertiary tumor transplantation experiments were carried out with P3C4 and P1E2 secondary tumor–derived cells, 1,000 cells again reinitiated tumor development in 90% of injections (Table 1; Fig. 3*C*).

When the P1E2 holoclone tumor–derived PC3-GFP cells were replated in clonal cultures, within ~1 week, all three types of clones were observed (Supplementary Fig. S2*B*), suggesting that holoclones could regenerate (or maintain) the clonal heterogeneity *in vivo*. These serial tumor transplantation experiments and replating assays provide concrete experimental evidence that PC3 holoclones contain stem-like tumor cells that could initiate serially transplantable tumors.

Holoclones express high levels of stem and progenitor cell markers CD44, $\alpha_2\beta_1$ integrin, and β-catenin. To further characterize stem cell–associated properties in holoclones, we immunophenotyped bulk-cultured PC3-GFP (Fig. 4*A*) or PC3 (not shown) cells for the expression of three stem and progenitor cell markers—CD44, $\alpha_2\beta_1$ and β-catenin. Our previous work has shown that the $CD44^+$ and $CD44^+\alpha_2\beta_1^+$ prostate cancer cell populations are enriched in tumorigenic and metastatic CSCs, whereas the $\alpha_2\beta_1^+$ prostate cancer cells most likely mark fast-proliferating tumor progenitors (28, 29). Furthermore, β-catenin is preferentially expressed in the $CD44^+$ prostate CSCs (28). As shown in Fig. 4*A*, holoclones in bulk-cultured PC3-GFP cells expressed high levels of all three molecules, whereas paraclones showed barely detectable expression of the three markers. Staining of regular PC3 cells (not shown) or holoclone-derived PC3-GFP cultures (Fig. 4*B*) revealed similarly differential expression patterns of these three stem cell markers. Importantly, meroclone P2H3-derived cells, which, as expected, did not form holoclones, expressed little CD44 and $\alpha_2\beta_1$ (Fig. 4*C*) or β-catenin (data not shown). These immunostaining results provide direct experimental evidence that PC3 cell holoclones, but not meroclones and paraclones, contain stem-like cells. The fact that all three markers are expressed in essentially all cells in the holoclones suggests that these three molecules are probably expressed in both cancer stem and progenitor cells.

In summary, we addressed in this study the critical question of whether epithelial cancer cell holoclones may contain stem-like cells. Through clonal analyses, serial passaging *in vitro*, serial tumor transplantation experiments, replating assays, and immunophenotyping, we provide unequivocal evidence that PC3

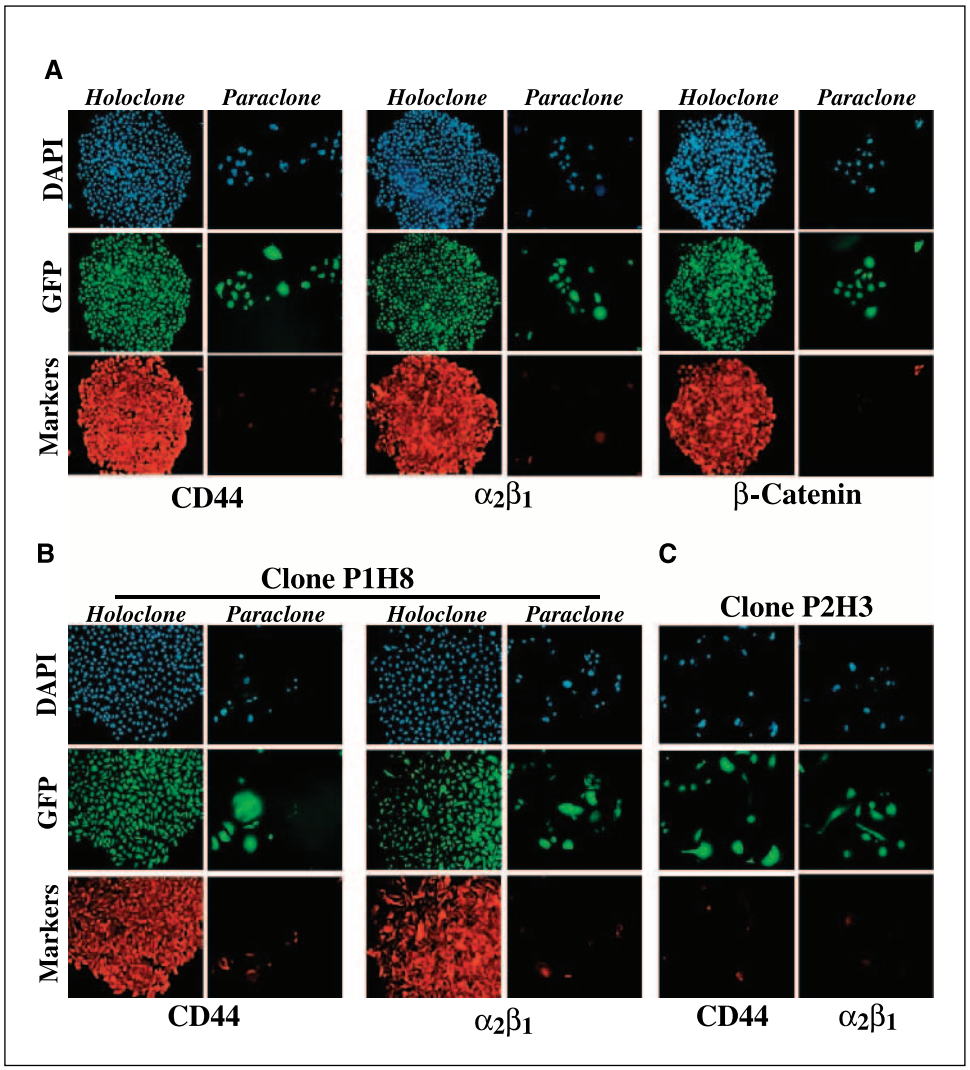

Figure 4. PC3 cell holoclones express high levels of stem and progenitor cell markers. Bulk-cultured PC3-GFP cells (*A*), holoclone P1H8 (*B*)–, or meroclone P2H3 (*C*)–derived cells were plated on glass coverslips and stained for CD44, $\alpha_2\beta_1$, and/or β-catenin using monoclonal antibodies. Original magnifications, ×100.

prostate cancer cell holoclones contain self-renewing cancer cells that could initiate serially transplantable tumors. Because tumor regeneration in serial transplantation experiments is the current gold standard for defining CSCs (31, 34), our results suggest that PC3 holoclones contain putative CSCs. In support, PC3 cell holoclones, but not meroclones and paraclones, express three well-established stem and progenitor cell markers. This suggestion is also consistent with primary keratinocyte holoclones containing normal stem cells (2–4). The observations made herein are unlikely restricted to only PC3 cells as Du145 holoclones can also be serially passaged and sustain long-term propagation (31) and many other epithelial cancer cells form holoclones that also express CSC markers such as CD44 (30). Future work will focus on devising ways to enrich for stem-like cells in cancer cell holoclones and then prospectively purify them and characterize their biological properties.

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