Stem and Progenitor-Like Cells Contribute to the Aggressive Behavior of Human Epithelial Ovarian Cancer

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Abstract

The cellular mechanisms underlying the increasing aggressiveness associated with ovarian cancer progression are poorly understood. Coupled with a lack of identification of specific markers that could aid early diagnoses, the disease becomes a major cause of cancer-related mortality in women. Here we present direct evidence that the aggressiveness of human ovarian cancer may be a result of transformation and dysfunction of stem cells in the ovary. A single tumorigenic clone was isolated among a mixed population of cells derived from the ascites of a patient with advanced ovarian cancer. During the course of the study, yet another clone underwent spontaneous transformation in culture, providing a model of disease progression. Both the transformed clones possess stem cell–like characteristics and differentiate to grow in an anchorage-independent manner *in vitro* as spheroids, although further maturation and tissue-specific differentiation was arrested. Significantly, tumors established from these clones in animal models are similar to those in the human disease in their histopathology and cell architecture. Furthermore, the tumorigenic clones, even on serial transplantation continue to establish tumors, thereby confirming their identity as tumor stem cells. These findings suggest that: (*a*) stem cell transformation can be the underlying cause of ovarian cancer and (*b*) continuing stochastic events of stem and progenitor cell transformation define the increasing aggression that is characteristically associated with the disease. (Cancer Res 2005; 65(8): 3025-9)

Introduction

A hypothesis based on the similarities between normal and tumor stem cells postulates that the former could themselves be targets of stochastic transforming mutations or give rise to a hierarchy of progenitors and differentiated cells that make up the tumor mass (1). Such approaches have been extensively investigated in leukemia (2, 3) and indicate that along with the establishment of a hierarchy, the transformed leukemic blast stem cells may be differentiation arrested (4).

An understanding of the emergence and organization of solid cancers at the cellular level has only recently been addressed, and has resulted in the prospective isolation and characterization of cancer stem cells in breast (5) and brain (6, 7). Ovarian cancer is an extremely aggressive disease associated with a lack of early symptoms, rapid progression to peritoneal metastases, and poor prognosis for patients. At advanced stages of the disease, the ascites gets enriched with tumor cells that form multilayered spheroids—the cellular nature of which has not yet been defined (8). The present study constitutes the first report of the establishment, isolation, cloning, and propagation of the cellular content of ovarian multilayered spheroids. We further show that the multilayered spheroid cells display heterogeneity in their clonogenic, tumorigenic, and invasive properties and also exhibit stem and progenitor-like qualities.

Materials and Methods

Cell culture and clone derivation. The present study was approved by the Bio-Ethics Committee of National Centre for Cell Science and informed consent was obtained from the patients. Cells from the ascites sample from a 63-year-old patient undergoing surgery for the removal of ovarian tumor diagnosed as a malignant grade IV serous adenocarcinoma were isolated using standard procedures. The scheme developed for clone isolation is depicted in Supplementary Fig. 1*A*. Cultures were incubated in a humidified tissue culture incubator at 37°C, 5% CO_2 atmosphere and were fed with fresh medium-MEM (E) supplemented with 5% fetal bovine serum and 1% nonessential amino acids every 4 days. Initially, the multilayered spheroids did not adhere to the surface of culture dishes and were collected at each passage by centrifugation, resuspended in fresh medium and plated out, continuing until substrate adherence was achieved. Sixty-five individual sublines were isolated from an attached spheroid (Supplementary Fig. 1*B*), following a 1:25 split ratio for marking single cells and picking up the clones developed with cloning cylinders.

Determination of capacity for anchorage-independent growth *in vitro*. Standard clonogenicity and spheroid generation assay procedures were followed. Briefly, for colony formation, subconfluent cultures were harvested, suspended in 0.5% agarose at a concentration of 5×10^3 cells per milliliter, and seeded in 35 mm plates containing a basal layer of 1% agarose. Colonies were counted after 2 weeks under a microscope at 10× magnification. For sphere formation, 10^6 cells were suspended in 3 mL medium (plain MEM without fetal bovine serum), and plated in bacteriologic dishes to prevent adherence. Spheres were counted at 3 and 6 days after plating. All experiments were done in triplicate.

Evaluation of tumorigenicity in nude mice by subcutaneous and metastases by intraperitoneal injections. Animal experimentation was done in accordance with the rules and regulations of the National Centre for Cell Science Animal Ethics Committee. Tumorigenicity was assessed by injecting A2 and A4-T cells (1×10^6 cells per milliliter in PBS) into nude mice, either s.c. into the thigh or i.p. Animals were kept in pathogen-free conditions and tumor growth was assessed every 3 days for 2 months or until the tumor diameter was 1 cm, whereupon animals were sacrificed to dissect out tumors. Mice given i.p. injections were observed for lethargy and poor appetite, and were sacrificed when their movements were compromised due to accumulation of ascites in the abdomen. To determine the extent of metastases and infiltration of the cells, samples of the omentum, stomach, intestine, liver, lungs, kidney, and heart from the experimental animals were collected and fixed for histologic examination (H&E staining), or collagenase digested, washed thrice with PBS, filtered through 40 μm cell strainers and reinjected into nude mice (1×10^6 cells per milliliter in PBS) to determine sequential tumorigenicity.

Note: A.M. Mali and N.K. Kurrey contributed equally to this paper.

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

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Immunofluorescence staining. Cells were grown on glass coverslips for 48 hours, fixed in ice-cold methanol or 4% paraformaldehyde at 4°C for 10 minutes, washed with PBS and blocked at room temperature for 30 minutes with 1% normal goat serum. Primary antibody incubation followed at room temperature for 30 minutes. After washing thoroughly with PBS, cells were further probed with FITC/PE-tagged secondary antibody. Coverslips were mounted in antifade solution and viewed under confocal microscope (Carl Zeiss, Jena, Germany). The list of primary antibodies used and their specific dilutions can be provided on request.

RNA isolation and reverse transcription-PCR. Total RNA was extracted from cells using TRIzol. RNA was reverse-transcribed using cDNA Synthesis Kit, (Invitrogen, Carlsbad, CA). cDNA was amplified using 1 μL of the reverse transcriptase reaction products in 25 μL with 10 pmol of the primers for 35 cycles. Each cycle consisted of 30 seconds of denaturation at 94°C, 30 seconds of annealing and 60 seconds of extension at 72°C. The PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining. The primer sequences used for cDNA amplification (forward and reverse) can be provided on request. β-Actin was used as the internal control in all reactions.

Statistical analysis. Results of experimental points obtained from multiple experiments are reported as mean ± SD. The significance of differences in mean values were determined using Student's *t* test.

Results and Discussion

The stringent, low-density culture system gave rise to 65 clones, 19 of which were spontaneously immortalized (designated A2, A3, A4, A5, B2, B7, C4, D4, F1, F2, F3, F4, F5, E1, E2, G5, G7, H1, and H4). Further work with these clones was carried out with cultures between the 8th and 12th passages after cloning. The remaining clones underwent senescence within 4 to 5 weeks of cloning, in a manner similar to that of the adherent populations initially obtained from the sample (Supplementary Fig. 1*A*). This senescence could be attributed to either, their being derived from committed non-immortalized progenitors, or due to the selective pressure imposed by an unvarying culture regime.

The immortalized clones showed variations in morphology and growth rates (Fig. 1*A*). The A2 clone had the shortest doubling time of 18 hours, whereas the others displayed doubling times ranging from 34 to 47 hours. A2 cells grew as small, spindle-shaped cells that were not contact-inhibited, could form foci, and grow to high densities in culture. A4 cells were similar in morphology to A2 cells, but lacked their growth potential at early passages. Since all cell cultures were maintained at identical growth conditions in the same culture milieu, their varying growth kinetics could be attributed to cell intrinsic mechanisms.

Semiquantitative reverse transcription (RT)-PCR was carried out in 10 representative clones in order to identify the nature of the isolated cells. Coexpression of cytokeratin 18 and vimentin was evident in all the clones (Fig. 1*B*). Similarly, the growth factor receptors c-met and epidermal growth factor receptor were up-regulated as was the surface adhesion molecule CD44. Cells also expressed E-cadherin; an exception being the A4 clone in which E-cadherin transcripts were not detected (Fig. 1*C*). Correspondingly, Snail, a known mediator of epithelial-mesenchymal transition through transcriptional repression of E-cadherin in ovarian cancer (9), was expressed in the A4 cells and to a lesser extent in the B7 clone. These expression patterns indicate the mesothelial nature of the cells (10)—which is in agreement with the current understanding of epithelial ovarian carcinoma resulting from the transformation of a primitive mesothelium, i.e., the ovarian surface epithelium (11).

Another member of the Snail family—Slug—was also seen to be expressed at comparable levels in the clones without the expected association of E-cadherin down-regulation (considered a marker

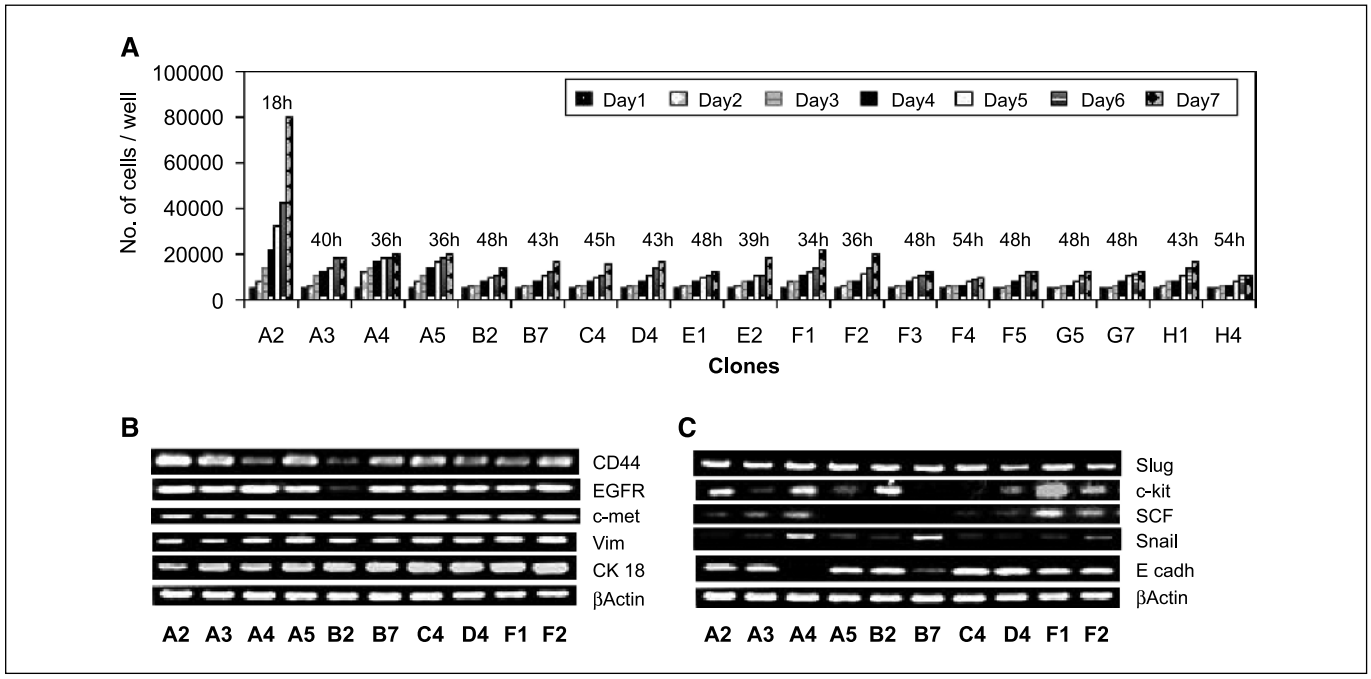

Figure 1. Isolation and characterization of tumorigenic clones from a heterogeneous population in the ovarian multilayered spheroids. *A,* growth kinetics of the 19 immortalized clones isolated from the multilayered spheroids. Cell proliferation assays were carried out over a period of 7 days. Figures at the top of each set of columns indicates the doubling time of that clone. The experiment was conducted in triplicate and average number of cells was plotted against time (days); *B,* RT-PCR analyses of expression of cytokeratin 18, vimentin, c-met, epidermal growth factor receptor, CD44, and mRNA in 10 selected clones that indicate the mesothelial nature of the isolated clones; *C*, RT-PCR analyses of expression of E-cadherin, Snail, SCF, c-kit, and Slug mRNA in the 10 clones that indicate heterogeneity of expression of epithelial-mesenchymal transition and survival-associated molecules. β-Actin mRNA expression was used as an internal control in (*B*) and (*C*).

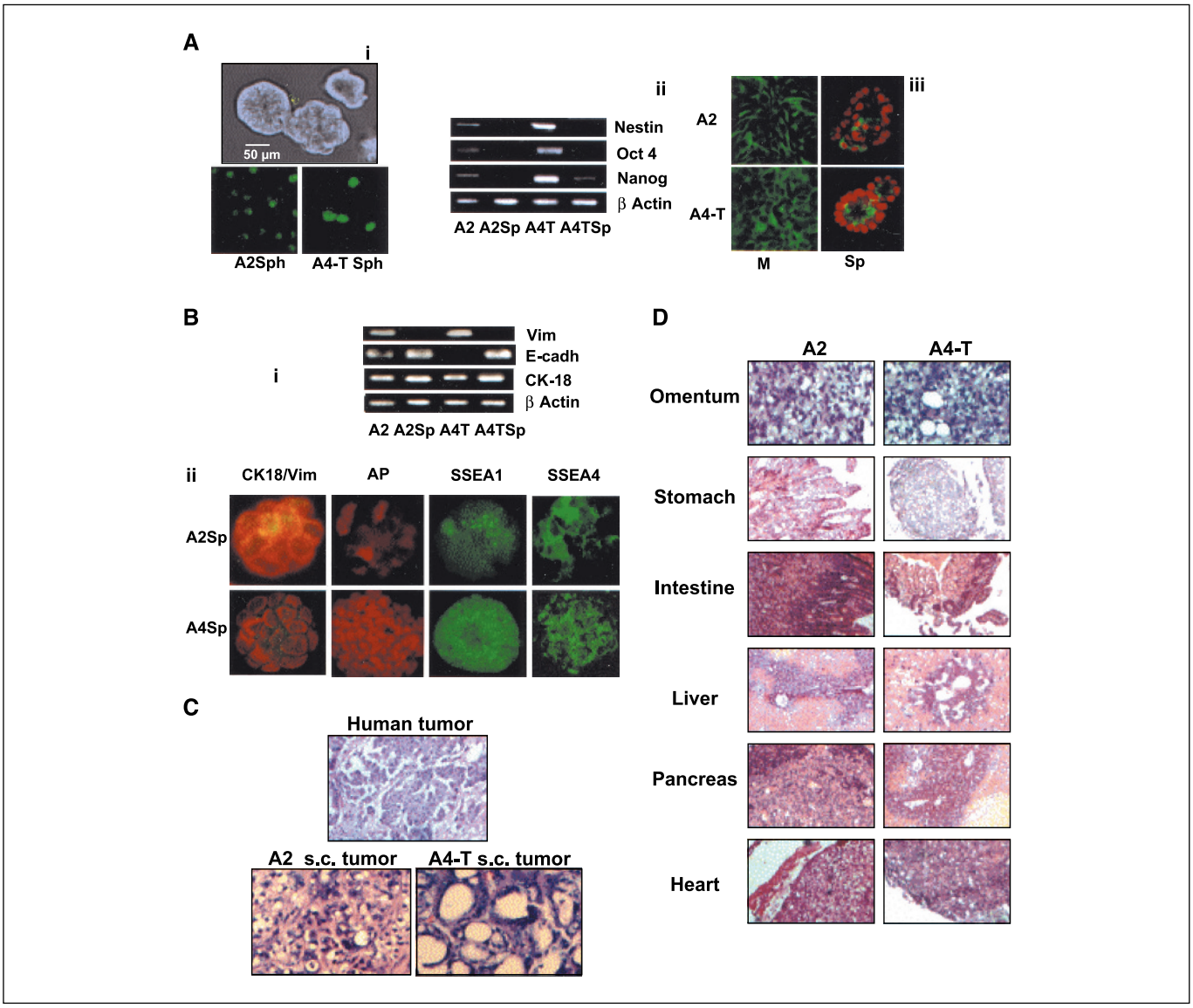

Figure 2. Spheroid and tumor characterization. *A,* (*i, top*) phase contrast image of typical spheroids generated in culture; (*bottom*) Ki-67 expression (immunofluorescence) indicating self-renewing A2 spheroids (A2Sp-X100) and A4-T spheroids (A4TSp-60X); (*ii*) RT-PCR expression of Nanog, Oct-4, and Nestin mRNA derived from monolayers (A2 and A4-T) and spheroids (A2Sp and A4-TSp). β-Actin mRNA expression used as an internal control; (*iii*) Nestin expression (immunofluorescence) in monolayers (M-X10) and spheroids (Sp-X20); *B* (*i*) RT-PCR analyses of expression of cytokeratin 18, vimentin, and E-cadherin mRNA in monolayers and spheroids. β-Actin mRNA expression used as an internal control; (*ii*) immunofluorescent staining of cell-specific markers (×60). Ck8/18 (*red*) and vimentin (*green*), alkaline phosphatase expression, stage-specific embryonal antigen 1 and 4 expression; *C,* representative photomicrographs of H&E stained sections (×4) of patient's tumor (*top*) and s.c. tumors derived from A2 and A4-T cells in nude mice (*bottom*). A4-T tumors seem to be more differentiated than A2 tumors initiated at the same time; *D,* representative photomicrographs of H&E stained sections (×4) tumor metastases to omentum, stomach, intestine, liver, pancreas, and heart.

for epithelial-mesenchymal transition). This indicated that Slug, in ovarian cancer, may have alternative function(s). Recently, Slug has been described as an important mediator of hematopoietic stem cell survival through the c-kit-SCF pathway (12). Our analysis revealed that c-kit and SCF are differentially expressed in the 10 clones—ranging from total absence to high-level expression (Fig. 1*C*) from which a specific definitive role for Slug through the same pathway cannot be arrived at. Nevertheless, the varying expression patterns extend the earlier observation of differences in growth patterns of the clones to their differential levels of expression of molecules known to be associated with epithelial-mesenchymal transition and cell survival.

In exploring the capacity for anchorage-independent growth of the 19 clones, only the A2 cells expressed this potential and formed 593 ± 72.6 (mean ± SD) colonies in soft agar within 14 days of culture. After ~20 passages, A4 cells (thenceforth termed as A4-T cells) gave indications of continuing mutagenesis, evinced through an increased proliferation rate and clonogenicity-forming 1,915 ± 142.66 soft agar colonies within 10 days by the 25th passage with an average larger colony size than that of the A2 colonies in soft agar (Supplementary Figs. 2*A*, *B*, and *C*, respectively). When grown in suspension, only cells of the tumorigenic clones, A2 and A4-T gave rise to organized spheroids (Fig. 2*A*, *i*, *top*) with A4-T showing a much higher spheroid-forming capacity than A2 (data not shown). The spheroids are self-renewing and have been maintained for over 15 to 20 passages *in vitro*—a capacity that correlates with a high level expression of Ki-67, a proliferation marker (Fig. 2 *A*, *i*, *bottom*).

Table 1. *In vivo* tumorigenicity of A2, A4, and A4-T cells

Cells	Site of injection	Tumor formation	Ascites formation	Metastases	Mortality	Tumor formation on serial transplantation
A2 (P8-P10)	s.c.	9/9	—	—	—	3/3
	i.p.	4/4	2/4	4/4	1/4	—
A4 (P8)	s.c.	0/2	—	—	—	—
A4-T (P25-P28)	s.c.	6/6	—	—	—	3/3
	i.p.	5/5	3/5	4/5	2/5	3/3

The A2 and A4-T cells were further analyzed for expression of specific markers known to be associated with stem and/or progenitor cells including Nestin, a progenitor marker (13), and Oct4 and Nanog, transcriptional determinants essential for the maintenance of an undifferentiated state (14). Nestin and Nanog were distinctly expressed in A2 as well as A4-T monolayers, and were reduced on differentiation into spheroids (Fig. 2 *A*, *ii* and *iii*), whereas Oct4 was expressed in A2 monolayers but was totally absent in spheroids. The expression of these three markers indicates a possible multipotent nature of the A2 and A4-T clones.

Tissue-specific differentiation is a unique characteristic of adult stem cells (15). Unfortunately, the development of cell lineages from adult stem cells in the ovary is not as well delineated as in other tissues like bone marrow. Although the existence of mesothelial and germ line stem cells in the postnatal adult mammalian ovary have been implied (16, 17), their isolation and characterization has not been achieved as yet. Since spheroid formation itself represents a differentiation event, we probed for the expression of markers in spheroids that could indicate either differentiation into ovarian surface epithelium (cytokeratin 18 and vimentin), granulosa (cytokeratin 18 and E-cadherin), or germ cells (alkaline phosphatase, stage-specific embryonal antigens 1, 3, and 4, and tumor rejection antigen 1-60 and 1-81). Indeed, differentiation along these three ovarian lineages could be identified (Fig. 2 *B*, *i* and *ii*), albeit the fact that germ cell differentiation was aberrant (alkaline phosphatase, stage-specific embryonal antigens 1 and 4 were expressed, whereas stage-specific embryonal antigen 3 and tumor rejection antigens 1-60 and 1-81 could not be detected). Tissue-specific differentiation thus seems to be incomplete and may be blocked in a manner akin to the maturation arrest during blast crisis (18) or in mammary tumor spheroids (19).

The *in vivo* correlate of the *in vitro* clonogenic potential of the candidate tumor stem cells was further assessed. Both clones formed tumors and underwent metastasis in nude mice, with A4-T cells being more aggressive than A2 cells (Table 1). The average latency periods in case of A2 cells for s.c. tumor formation (~ 1 cm diameter) and i.p. ascites formation were 28 and 55 days, respectively, whereas those for A4-T cells were 7 and 18 days, respectively. The tumors were classified as serous adenocarcinomas, and exhibited a high degree of similarity with the primary tumor in the patient. An appreciable difference was that A2 tumors were relatively undifferentiated as seen from their solid pattern and limited gland formation in comparison with the A4-T tumors; from these differences in the cell architecture, it was inferred that the A4-T tumors were more similar to those in the human disease (Fig. 2*C*).

In the i.p. mode of introduction into nude mice, along with ascites formation, omental growth and peritoneal metastases were evident in both clones. The pathology of the omental mass was similar to that of the s.c. tumors (Fig. 2*D*). In the stomach and intestine, tumors developed mainly on the serosal surface, showed atypical nuclei at high frequency and progressed to form papillary serous adenocarcinomas in both the clones. In A4-T tumors, tumor infiltration to the other layers was also evident, whereas abnormal mitoses were more frequent in the A2 intestinal tumors. The extent of tumor infiltration was higher in the pancreas and liver, which showed moderate differentiation. Tumors formed in the heart were relatively solid and undifferentiated, with growth being observed within the lumen and attached to the endocardium. No signs of metastases to the kidneys, lungs, or spleen could be detected.

To conclusively show that the clones A2 and A4-T are indisputably tumorigenic stem cells, s.c. tumor–derived cells were further reinjected into mice. Successful serial tumor formation confirmed the existence of cells in both the clones that were able to propagate the tumor even in consecutive generations. Such cells in the tumor mass are retained due to stem cell renewal mechanisms, despite overall tumor differentiation and progression, and provide strong evidence of the existence of a small fraction in the tumor capable of driving tumorigenesis.

In conclusion, the two transformed clones, A2 and A4-T, represent tumor stem cells because (*a*) they self-renew and are clonogenic, (*b*) differentiate *in vitro* to form organized spheroids in suspension, (*c*) express multipotency and tissue-specific differentiation markers, (*d*) express self-renewal mechanisms *in vivo* (sequential tumorigenicity), and (*e*) undergo *in vivo* differentiation to produce a disease similar to that in the patient.

The data thus provides evidence of the origin of ovarian cancer from the transformation of a stem cell that has extensive self-renewal capabilities and also undergoes differentiation (A2 clone). Continuing stochastic mutagenic events in other stem/progenitor populations (evinced in the spontaneous transformation of A4 to A4-T), further suggests a mechanism for the increased aggressiveness during tumor progression that is usually associated with ovarian cancer. Further characterization of the tumorigenic populations will allow for the identification of molecules expressed in these cells that could serve as targets to eliminate this fraction of cancer cells that can rapidly develop the critical tumor cell mass. Consequently, defining the unique properties of these tumor stem cells remains a high priority for developing early diagnostic and effective therapeutic strategies.

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