Side Population Cells Isolated from Mesenchymal Neoplasms Have Tumor Initiating Potential

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Abstract

Although many cancers are maintained by tumor-initiating cells, this has not been shown for mesenchymal tumors, in part due to the lack of unique surface markers that identify mesenchymal progenitors. An alternative technique to isolate stem-like cells is to isolate side population (SP) cells based on efflux of Hoechst 33342 dye. We examined 29 mesenchymal tumors ranging from benign to high-grade sarcomas and identified SP cells in all but six samples. There was a positive correlation between the percentage of SP cells and the grade of the tumor. SP cells preferentially formed tumors when grafted into immunodeficient mice, and only cells from tumors that developed from the SP cells had the ability to initiate tumor formation upon serial transplantation. Although SP cells are able to efflux rhodamine dye in addition to Hoechst 33342, we found that the ability to efflux rhodamine dye did not identify a population of cells enriched for tumor-initiating capacity. Here, we identify a subpopulation of cells within a broad range of benign and malignant mesenchymal tumors with tumor-initiating capacity. In addition, our data suggest that the proportion of SP cells could be used as a prognostic factor and that therapeutically targeting this subpopulation of cells could be used to improve patient outcome. [Cancer Res 2007;67(17):8216–22]

Introduction

Solid tumors are composed of a heterogeneous population of cells. These cells have different *in vitro* proliferative capacities; only a minority have the ability to initiate tumor formation in immunodeficient mice. This observation led to the concept of cancer stem cells (CSC), which have the ability to self-renew and differentiate. By manipulating these characteristics, CSCs have been postulated to be responsible for driving the growth of tumors and the recurrence of neoplasms after therapy (1, 2). Although these cells have been identified in a variety of malignancies, such as hematologic, neural, and epithelial cancers, they have not been identified in neoplasms of mesenchymal origin. As such, cell surface markers used in these tumors may not be successful for the isolation of these CSCs from mesenchymal neoplasms (3–11). Furthermore, there are no unique markers for mesenchymal stem cells. Fortunately, other properties of stem cells can be used to isolate cells with progenitor characteristics.

One such property is the ability of stem cells to efflux chemotherapeutic drugs and certain dyes, such as Hoechst 33342 (12, 13). This feature is conferred partly by high levels of expression of ATP-binding cassette (ABC) transporter proteins on primitive progenitor cells (14, 15). During flow cytometry analysis, negatively stained cells fall to the "side" of the majority; hence, they are commonly called side population (SP) cells (16). They enrich for progenitor cells from a variety of tissue types (17–20); furthermore, data have proved that some cancer cell lines and primary tumors contain SP cells. It is the SP cells that confer tumorgenicity of the tumor or cancer cell line (21, 22). This technique provides an alternative means to isolate progenitor cells other than through the use of specific surface markers and may be one way to identify putative tumor–initiating cells.

Primary musculoskeletal tumors are of mesenchymal origins. They range from benign lesions, such as the soft tissue aggressive fibromatosis (also called desmoid tumors), to aggressive malignancies, such as synovial sarcomas. Mesenchymal progenitors express a variety of markers, such as Stro-1, CD105, and CD44; however, to date, no marker or pattern of markers universally defines a consensus phenotype for a mesenchymal stem cell. This has hampered research into the cell of origin of these lesions (23–26). Recent *in vitro* evidence suggests that mesenchymal tumors may contain cells with stem-like characteristics. Cells within these tumors had the capacity to form spheres similar to those formed by neural stem cells. Furthermore, these spheres preferentially expressed genes that are involved in regulating stem cell fate (27). Despite this, the ability to identify and isolate tumor-initiating cells (TIC) within mesenchymal tumors has proved to be elusive.

The initiation and progression of these tumor types is poorly understood. These lesions can have a high rate of recurrence, suggesting that a small population of cells has the potential to escape treatment and may have TIC characteristics (28). SP cells, which are enriched with TICs, efflux dye through a mechanism that is similar to that used by chemotherapeutic resistant cells. Therefore, TICs may be relatively resistant to chemotherapeutic drugs and may confer the malignant phenotype to tumors (13, 29, 30). As such, these cells could be responsible for sarcoma recurrence after chemotherapy. Here, we report, for the first time, the identification of SP cells from mesenchymal tumors. These cells have the capacity to initiate tumor formation. Further investigation of these TICs may lead to the development of more effective and efficient targeted treatment modalities.

Materials and Methods

Primary tumors. Twenty-nine mesenchymal tumors were processed at the time of surgical excision. Local ethical approval was obtained for all

Note: C. Wu and Q. Wei contributed equally to this work.

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tissue samples collected. The samples included seven aggressive fibromatosis, five osteosarcomas, three chondrosarcomas, three synovial sarcomas, two leiomyosarcoma, four malignant fibrous histocytomas, one myxoid liposarcoma, one pleomorphic liposarcoma, one dermatofibrosarcoma protuberans, one myxoid chondrosarcoma, and one chordoma. Primary tumor samples were manually minced, and all visible clumps were removed. Enzymatic digestion followed at 37°C for 45 min with constant rotation using 10 mg/mL of collagenase IV (Worthington), 2.4 units/mL Dispase (Becton Dickinson), 0.05% trypsin (Wisent). Further manual dissociation was done by passing the cell slurry through an 18-gauge needle. Cells were then centrifuged at 1400 rpm for 5 min and washed thrice in PBS. After washing, cells were strained through 70-μm filters to remove remaining clumps. Collected cells were plated in α-MEM supplemented with L-glutamine and containing 18% fetal bovine serum (FBS; Wisent) and cultured at 37°C with 5% CO_2 in a humidified chamber until subjected to fluorescence-activated cell sorting (FACS).

Pathology. Formalin-fixed, paraffin-embedded samples were stained with H&E and observed in a blinded manner by an experienced pathologist. Tumors were graded into benign or malignant groups. Each tumor was subclassified according to the WHO classification and graded using the standard three-scale American Joint Commission on Staging (31–33). The grade 3 lesions included malignant fibrous histocytomas, synovial sarcomas, osteosarcomas, and one liposarcoma. The grade 2 lesions included one osteosarcoma, chondrosarcomas, and leiomyosarcomas. The grade 1 lesions included a liposarcoma, chondrosarcomas, chordomas, and dermatofibrosarcoma protuberans, myxoid chondrosarcoma.

Flow cytometry. Cells were trypsinized and resuspended in PBS supplemented with 2% FBS at a concentration of 1×10^6 cells/mL. For SP assays, cells were treated either alone with 2.5 μg/mL of Hoechst 33342 dye (Sigma) for 90 min at 37°C, or in combination with 50 μmol/L verapamil (Sigma), an inhibitor of ABC transporters. For Rhodamine-123 staining, 1×10^6 cells/mL were incubated with 0.1 μg/mL of Rhodamine-123 (Molecular Probes) for 30 min at 37°C. For analysis of Rhodamine-123 and Hoechst 33342 efflux activity, cells were initially incubated with 0.1 μg/mL of Rhodamine-123 for 30 min at 37°C, washed, and then resuspended in the same cell concentration with 2.5 μg/mL of Hoechst dye for 90 min at 37°C. Cells were counterstained with 1 μg/mL of propidium iodide (Molecular Probes), and nonviable cells were excluded from both analysis and sorting assays. To detect for SP, cells were analyzed by using a dual wavelength analysis (blue, 424–444 nm; red, 675 nm) after excitation with 350 nm UV light (MoFlow, Cytomation). SP and non-SP (NSP) cells were collected and injected into NOD/SCID mice. For Rhodamine-123 staining, 20% cutoffs were used to sort Rho^{high} and Rho^{low} fractions.

Cell transplantation into NOD/SCID mice. Sorted SP and NSP cells were collected, and cells were resuspended in PBS at concentrations ranging from 100 to 100,000 cells per 50 μL. Cells were then mixed with 50 μL of Matrigel (Becton Dickinson). We noted a high degree of cellular dispersion when mesenchymal cells are s.c. injected into NOD/SCID mice without stromal support. As such, we used Matrigel to act as a physical barrier to prevent the cellular dispersion and subsequent loss of injected cells. This step was critical for as few as 100 SP cells were s.c. injected; hence, there was a high likelihood of loss of the cells without Matrigel. This method has been shown to yield optimal tumor growth of mesodermally derived tumors (34). This cell-matrigel suspension was then s.c. injected into 8-week-old to 10-week-old NOD/SCID mice. Mice were observed for up to 12 weeks, after which they were euthanized and tumor formation was assessed. Tumors that formed were removed, and samples from each tumor were harvested for FACS and histology. For FACS, tumors were dissociated, as the primary tumors, and resorted into SP and NSP fractions. These secondary sorted cells were then reinjected into 8-week-old to 10-week-old NOD/SCID mice, and tumor formation was assessed after 12 weeks. Samples of tumors were also formalin-fixed and processed for histology.

Results

Mesenchymal tumors contain SP cells. We sought to determine whether we could isolate TICs from primary mesenchymal tumors based on the observation that stem-like cells have the ability to efflux Hoecsht 33342 dye (16). Cells from 29 primary mesenchymal tumors, ranging from locally invasive lesions to high-grade sarcomas, were incubated with the fluorescent dye Hoechst 33342. Cells were also incubated in the presence of verapamil, a chemical inhibitor of the ABC protein family of transporters, which inhibits the efflux of Hoechst (15). A distinct SP was found in all of the tumors examined except for one dermatofibrosarcoma protuberans (grade 1), one myxoid chondrosarcoma (grade 1), one chondrosarcomas (grade 1), one malignant fibrous histocytoma (grade 3), one leiomyosarcomas (grade 2), and a synovial sarcoma (grade 3). With the addition of verapamil, the presence of SP cells was abolished, indicating that dye efflux occurred, in part, through an ABC transporter–regulated mechanism (Fig. 1). The presence of an SP within these tumors raises the possibility that this represents a subpopulation of cells with tumor-initiating characteristics.

The proportion of SP cells correlates with aggressiveness of the tumor. Mesenchymal tumors are highly heterogeneous groups of neoplasms with varying levels of histologic and clinical aggressiveness (28). We observed a trend between the proportion of SP cells present in a given tumor and the relative aggressiveness of the tumor. In general, higher grade tumors had an increased prevalence of SP cells (Fig. 2). This suggests that the proportion of SP cells may be a predictor of patient outcome. Furthermore, SP cells may be responsible for the maintenance and propagation of mesenchymal tumors, giving the high-grade tumors a more aggressive behavior. However, numbers of individual tumor types were too small to determine if within a particular tumor type the percentage of cells sorting to the SP correlated with grade, aggressiveness, or clinical outcome.

SP cells have the capacity to form tumors upon serial transplantation in NOD/SCID mice. We tested the ability of SP cells to initiate tumor formation when grafted into NOD/SCID mice. Cells from one osteosarcoma, two malignant fibrous histocytomas, and one synovial sarcoma were stained with Hoechst 33342 dye and sorted into SP and NSP fractions. Both fractions were then s.c. injected into NOD/SCID mice. Initially, both SP and NSP fractions had the capacity to form tumors; however, significantly smaller numbers of SP cells formed tumors at a significantly higher frequency when compared with NSP cells (Table 1). The SP examined from each of the individual tumors showed the same enhanced ability to form tumors in NOD/SCID mice. Furthermore, although they formed from larger numbers of injected cells, tumors that formed from the NSP population were significantly smaller when compared with tumors from the SP fraction. The mean weight of tumors formed by SP cells was 0.19 ± 0.21 g; in comparison, the mean weight of tumors formed by NSP cells was 0.065 ± 0.063 g, $P < 0.005$. Tumors expressed human glyceraldehyde-3-phosphate dehydrogenase and had a nearly identical cytology to the primary sarcoma (Fig. 3).

To test for self-renewal (35), SP and NSP tumors were dissociated and cells from both tumors were restained with Hoechst 33342 dye. SP reanalysis showed that only cells derived from SP tumors were able to repopulate both SP and NSP fractions (Fig. 4*A*). The percentage of SP present from xenografted tumors was similar to that of the parental tumor cells, suggesting that SP cells had the ability to recapitulate the phenotypic distribution of the original tumor. When labeled with propidium iodide, there was significant decrease in viable cells in the tumors derived from the NSP compared with the cells derived from SP tumors (Fig. 4*B*). Viable

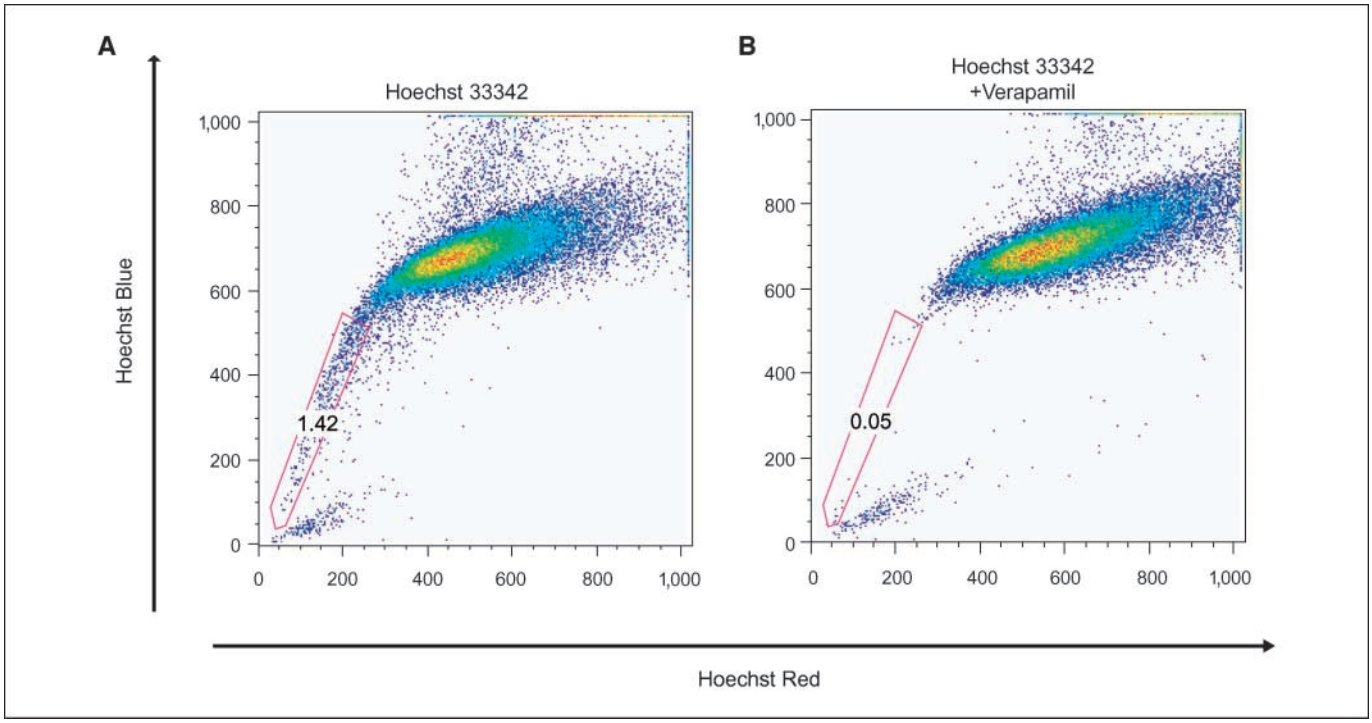


Figure 1. Mesenchymal tumors contain an SP. Cells from a representative grade 2 primary sarcoma were stained with Hoechst 33342 and analyzed by flow cytometry. *A,* the SP from a representative tumor. The SP cells are outlined and shown as a percentage of the total cell population. *B,* this cell population disappears in the presence of verapamil.

cells from NSP tumors had an increase in DNA content compared with the SP tumor cells, suggesting a higher mitotic rate (Fig. 4*C*). Furthermore, even after 32 weeks, NSP tumor cells injected into NOD/SCID failed to initiate tumor growth. Taken together, this suggests that NSP tumors represent a population of cells with only transient-amplifying potential.

To determine the tumorigenic potential of the cells from the xenograft tumors, we next serially transplanted SP and NSP cells

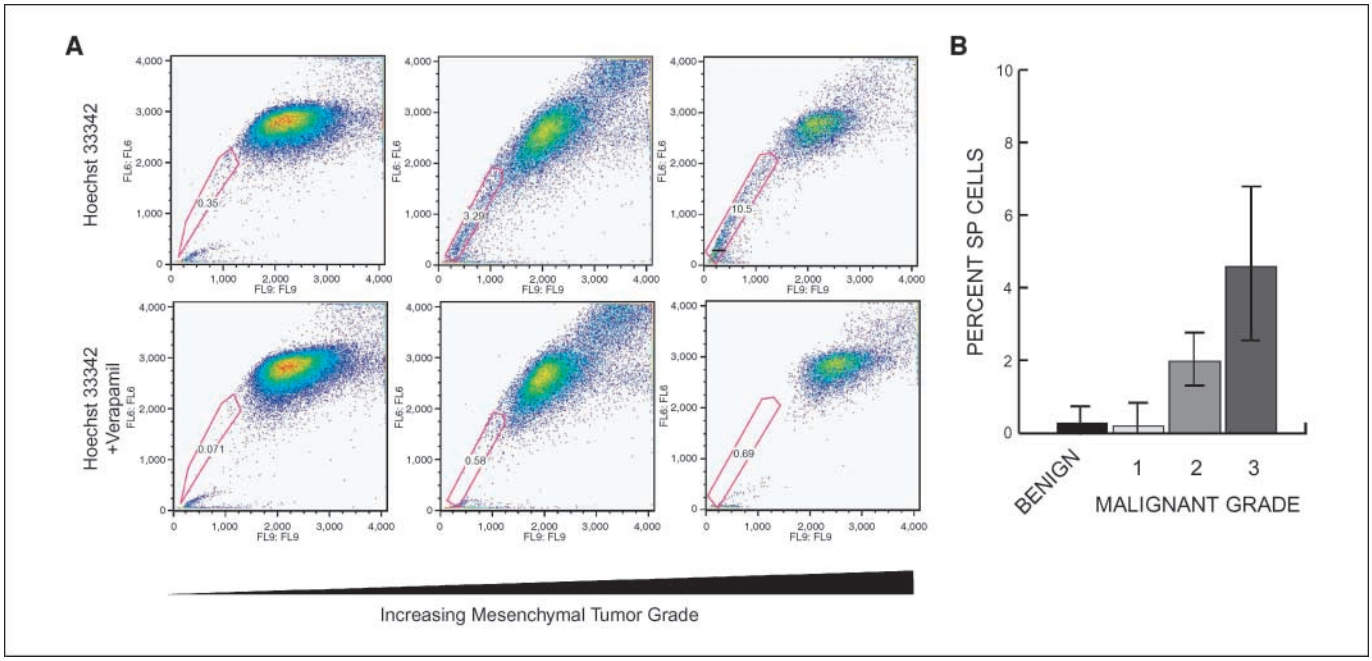


Figure 2. High-grade sarcomas have an increased prevalence of SP cells when compared with lower-grade lesions. *A,* SP analysis from a benign aggressive fibromatosis, a grade 2 leiomyosarcoma, and a grade 3 malignant fibrous histiocytoma. SP cells are outlined and shown as a percentage of the total cell population. *B,* mean and 95% confidence intervals for the percentage of SP cells from benign and various grade malignant lesions. A 95% confidence interval that does not cross the mean of a comparison is a statistically significant difference at $P < 0.05$. There are significantly higher proportions of SP cells in higher grade tumors.

Table 1. The proportion of tumors that formed from injection of various numbers of cells from each subpopulation into NOD/SCID mice

Cell type	Cell dose	No. primary mice with tumors/total no. injected	No. secondary mice with tumors/total no. injected	Total no. mice with tumors (%)
SP	1×10^2	9/14	3/8	12/22 (55)
	5×10^2	4/4	nt	4/4 (100)
	1×10^3	5/14	9/12	14/26 (54)
	1×10^4	8/10	2/2	10/12 (83)
	1×10^5	nt	nt	
NSP	1×10^2	1/12	0/8	1/20 (5)
	5×10^2	nt	nt	
	1×10^3	3/14	0/8	3/22 (14)
	1×10^4	1/10	1/10	2/20 (10)
	1×10^5	4/16	nt	4/16 (25)

NOTE: Primary mice were injected with cells sorted from the primary tumor, and secondary mice are injected with cells sorted from the tumor that developed in primary mice. SD cells from each tumor type examined formed tumors from the SP fraction at each cell number examined. In contrast, only in a few instances did NSP cells formed tumors in primary mice. Larger numbers of injected cells were required for the NSP cells to form tumors. The SP fraction from tumors that formed in the primary mice was able to form tumors in secondary mice, whereas in only one case did cells from the NSP form a tumor. None of the cells from the primary tumors that formed from NSP cells were able to form tumors in secondary mice.
Abbreviation: nt, not tested.

from the SP tumors and NSP tumors. In the secondary transplant, cells from NSP tumors did not initiate tumors in the NOD/SCID mice. Only the SP fraction from tumors from the initial SP fraction had the capacity to initiate tumor formation. Furthermore, as few as 100 SP cells resulted in the formation of tumors whereas 10,000 NSP cells failed to form tumors (Table 1). These secondary xenografted tumors had an identically histologic appearance to both primary xenografted tumors and to original parental tumors (Fig. 3). Hence, only tumors derived from SP cells can self-renew and differentiate in a manner that can be propagated through serial transplantation into NOD/SCID mice.

SP cells efflux Rhodamine-123. The phenomenon of dye efflux is not exclusive to Hoechst 33342. For instance, Rhodamine-123 efflux can be used to identify populations enriched for hematopoetic stem cells from mouse bone marrow. We next examined the ability of Rhodamine-123 efflux to recapitulate the results shown with Hoechst 33342 efflux. Staining with Rhodamine-123 resulted in a broad range of florescent uptake. Two populations were sorted based on gating of the upper and lower 20% of live cells, hereafter called Rho^{low} and Rho^{high}. Cells were also dual stained with both Rhodamine-123 and Hoechst 33342. We noted that the majority of SP cells did not stain highly for Rhodamine-123, whereas the staining pattern of NSP cells was identical to the general population of cells (Fig. 5*A*). Thus, SP cells have the capacity to efflux Rhodamine-123, and the two populations were not mutually exclusive. Rho^{low}, Rho^{high}, Rho^{high}/NSP, and Rho^{low}/SP cells were sorted and injected into NOD/SCID mice (Fig. 5*B*). Although both Rhodamine-123 populations failed to form tumors after 12 weeks, as few as 375 Rho^{low}/SP cells did initiate tumor formation in two out of two mice. This corresponded to the number of cells required to form tumors from SP cells, suggesting that the capacity to efflux Rhodamine-123 does not enhance the tumorigenic potential of SP cells.

Discussion

Here, we show for the first time that mesenchymal tumors contain a subpopulation of cells with tumor-initiating capacity,

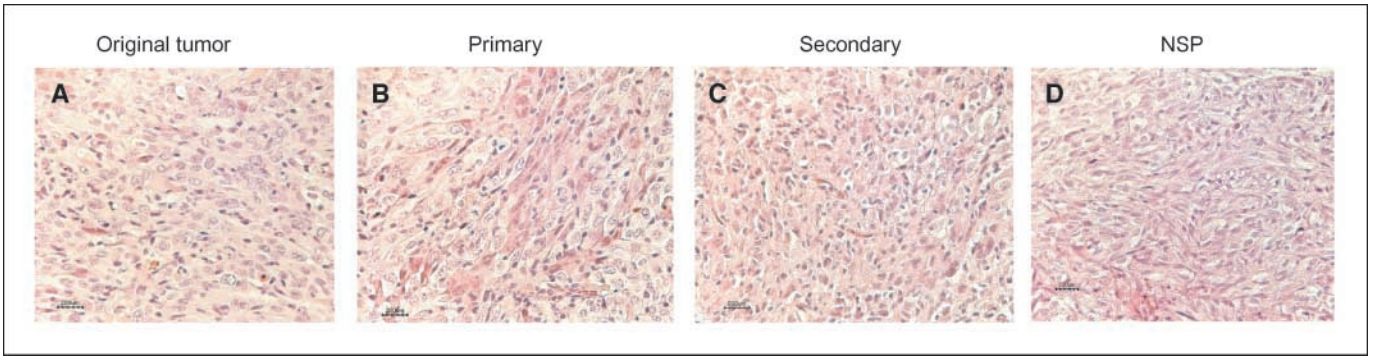


Figure 3. Histopathologic features of SP tumors. SP cells from parental (*A*), primary (*B*), and secondary (*C*) xenografted tumors were injected into NOD/SCID mice. After 12 wks, tumors were collected, formalin-fixed, paraffin-embedded, and stained with H&E. The grafted tumors (*B* and *C*) formed had nearly identical cytologic appearances to the primary tumor (*A*). A representative NSP tumor (*D*) also showed similar cytologic appearances to the primary tumor. A representative malignant fibrous histiocytoma. *Size bar*, 200 μm.

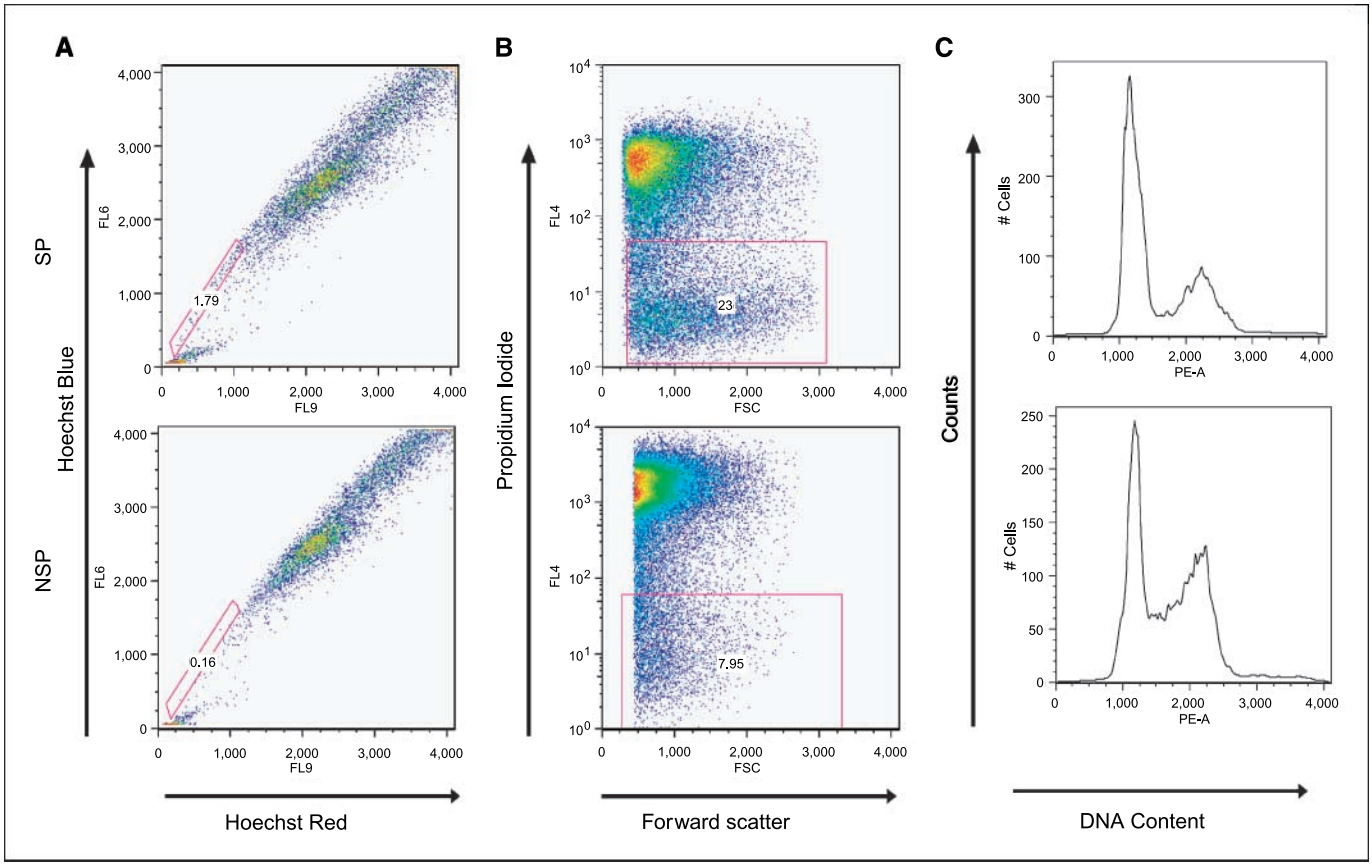


Figure 4. Characteristics of tumors derived from SP and NSP cells. *A,* cells derived from both SP and NSP primary xenograft tumors were reanalyzed for SP cells. Only SP tumors contained cells that had the capacity to reform both SP and NSP fractions. *B,* cell viability was determined by propidium iodide staining; 23% of cells derived from SP tumors were viable, whereas only 8% of cells derived from NSP tumors were viable. *C,* cells from NSP tumors had an increase in DNA content compared with the SP tumor cells, suggesting a higher mitotic rate.

which can be identified based on their exclusion of Hoescht 33342 dye. Intriguingly, there are higher proportions of SP cells in high-grade mesenchymal malignancies than in less aggressive benign lesions. Although TICs have been identified in hematologic, neural, and epithelial cancer (3–11), to date, this has not been proved for mesenchymal tumors whose origins differ from other solid tumors. Our data suggest that TICs are present in a broad range of benign and malignant neoplastic processes and, as such, are a general phenomenon in tumorigenesis.

The identification of TICs in mesenchymal tumors has proved to be more elusive than in tumors that originate from other tissue types, in part due to a lack of universally agreed upon marker, which selects and identifies mesenchymal progenitor cells (23, 25, 26). Other techniques to isolate stem cells include *in vitro* functional techniques. Indeed, recent work has shown that in an *in vitro* culture system, cells derived from mesenchymal tumors have the capacity to form spheres similar to those derived from neural stem cells (27). Although these "sarcospheres" preferentially express "stemness" genes compared with their adherent counterparts, it has not been shown that these cells have a preferential ability to form tumors when grafted into immunodeficient mice.

Given these obstacles, we chose to use Hoechst dye exclusion as an alternative method of stem cell isolation. Although this method has been successfully used to isolate stem-like cells in a variety of cell lines (21), it is infrequently used in primary tumors (22). We found that cells from most primary mesenchymal tumors contained an SP and that these cells have the capacity to initiate tumors when transplanted into immunodeficient mice. Furthermore, upon serial transplantation, only SP tumors had the ability to reinitiate the tumor in immunodeficient mice, suggesting that only this population is able to self-renew *in vivo*.

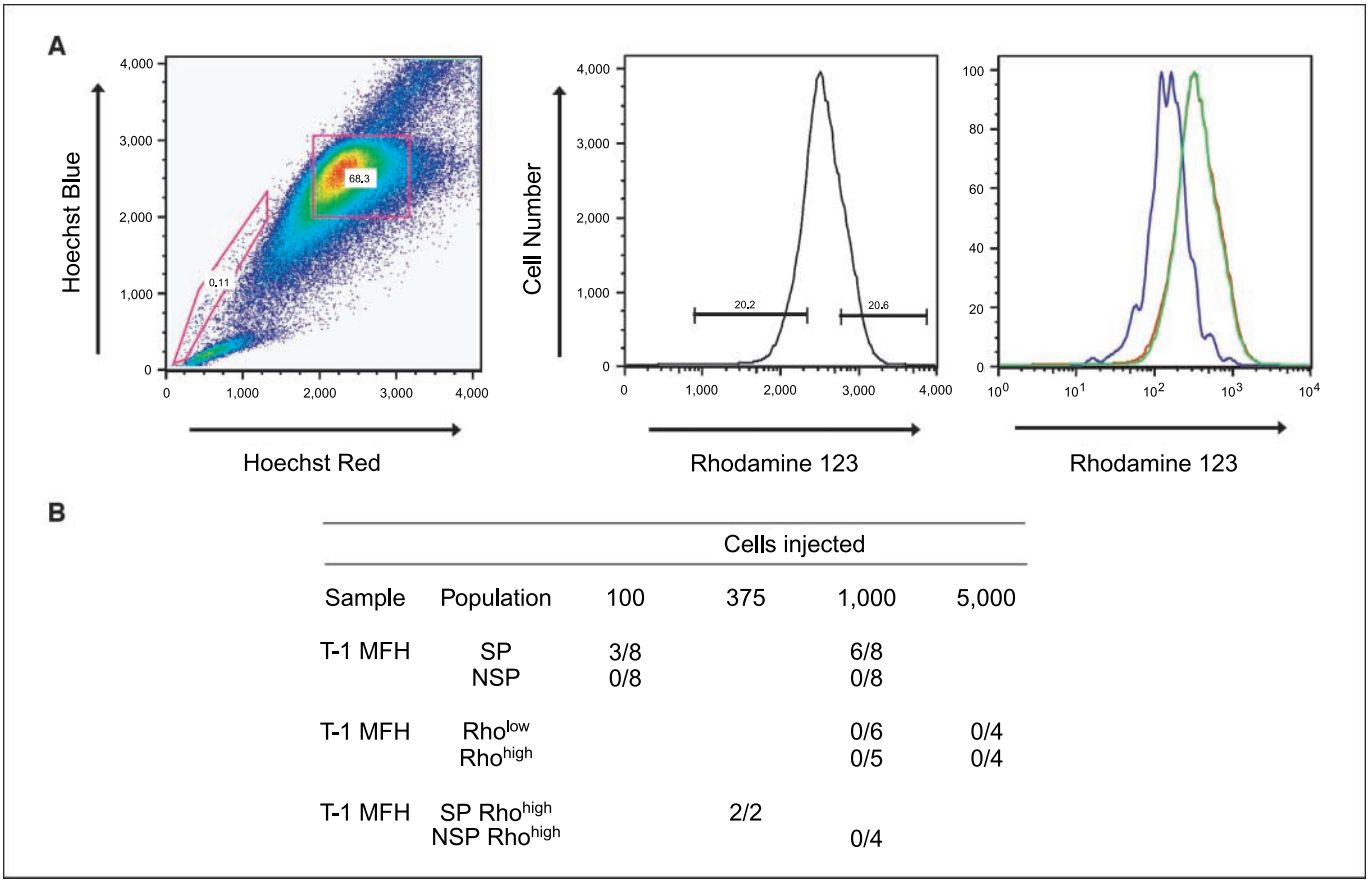
Large numbers of NSP cells also formed tumors in NOD/SCID mice, and others showed that in breast, prostate, and thyroid cancer cell lines, large numbers of NSP cells have the capacity to initiate tumor formation. It has been suggested that this finding is due to contamination of small numbers of SP cells in the NSP fraction (36–38). However, in our experiments, although cells from the NSP fraction were initially able to form tumors in NOD/SCID mice, when restained with Hoechst dye, they did not contain an SP fraction. They also exhibited high levels of cell death as shown by a high level of propridium iodide staining. Furthermore, analysis of DNA content showed that these cells have an increase in DNA content when compared with cells derived from SP tumors, suggesting that NSP tumor cells have an increased proliferation rate. However, we cannot exclude the possibility that differences in DNA content may be unrelated to cell proliferation, but rather they may be attributed to differences in genomic stability between the two populations of cells. Thus, an interesting alternative hypothesis may be that differences in DNA content may be a result of NSP cells having increased genomic instability resulting in an inability to self-renew. In contrast, SP cells may maintain DNA repair mechanisms, a key feature of stem cells resulting in a prolonged life span.

Importantly, NSP tumor cells were not able to initiate tumors after serial transplantation even after 32 weeks postinjection. This suggests that NSP cells are fundamentally different from their SP counterparts. Given these observations, it is possible that cells from the NSP fraction represent a more differentiated subpopulation, characterized by a short-term proliferative potential, such as a transient-amplifying cell. Taken together, this strongly suggests that Hoescht dye exclusion will enrich for cells with the capacity to divide asymmetrically and to self-renew, a key feature of stem cells.

As Hoechst dye exclusion selects for cells with the capacity to exclude dye via the expression of protein transporters (15, 39), we cannot exclude the possibility that SP cells are more mature tumor cells that have acquired the ability to increase the expression of genes responsible for this pumping mechanism. Such cells might also be resistant to chemotherapy and may be responsible for the relapse of disease (13). To address this issue, we isolated cells that effluxed the fluorescent dye Rhodamine-123. Unlike SP cells, neither Rho^{low} nor Rho^{high} cells had the capacity to initiate tumor formation; however, we cannot exclude the possibility that our gating strategy was too generous for enrichment of TICs and that, with increased stringency, there may be TICs in the Rho^{low} population. Despite this, our data suggest that the capacity to efflux materials may not the sole determinant of TICs because SP, but not Rho^{low}, cells had the capacity enriched for TICs. In any event, we have shown that SP cells are distinct from their NSP counterparts and are enriched for TICs. A more in-depth analysis of this population may provide important clues into sarcoma biology.

A striking result of our study is the correlation seen between the percentage of SP cells and the aggressiveness of the mesenchymal tumor examined. We found that benign, locally invasive, low-grade tumors had a low SP population whereas high-grade malignant tumors had high SP populations. The tumors that did not show an SP were from a variety of tumor grades. One confounding factor is that the complete absence of an SP may be due to technical factors, and in many of the lesions in which no SP was identified, only small numbers of cells were available from the biopsy for analytic analysis. Despite this, even including the small number of samples in each grade with no SP, there was still a substantial difference in the percentage of SP cells between the various grades. These data imply that SP is a predictor of patient outcome; however, prospective studies are needed to determine if this hypothesis will be proved to be correct.

The high proportion of SP cells in malignant tumors might also correlate with chemotherapeutic resistance, as this resistance may be due in part to the overexpression of transporter proteins which efflux drugs. As we have shown that SP cells have the capacity to efflux material and have enhanced tumorigenic potential when compared with NSP cells, it is interesting to hypothesize that the efflux mechanism plays a role in chemotherapeutic resistance.



B

Sample	Population	Cells injected: 100	375	1,000	5,000
T-1 MFH	SP	3/8		6/8	
	NSP	0/8		0/8	
T-1 MFH	Rho^{low}			0/6	0/4
	Rho^{high}			0/5	0/4
T-1 MFH	SP Rho^{high}		2/2		
	NSP Rho^{high}			0/4	

Figure 5. SP cells efflux Rhodamine-123. *A,* cells derived from a primary MFH sample were dual stained with both Hoechst 33342 and Rhodamine-123. Twenty percent cutoffs were used to sort Rho^{high} and Rho^{low} fractions. Shared profiles indicate SP cells (*blue*) have decreased Rhodamine-123 staining compared with the total population of cells (*green*). NSP cells (*red*) have a staining pattern similar to that of the total population of cells. *B,* cells were sorted into SP, NSP, Rho^{high}, Rho^{low}, SP/Rho^{low}, and NSP/Rho^{high} fractions, and varying cell numbers were injected into NOD/SCID mice. Tumor formation was assessed 12 weeks postinjection. Numbers indicate the number of tumors that formed/number of injections.

Perhaps, the expression of transporter proteins would correlate with both the outcome and the proportion of SP cells. Hoechst dye exclusion is conferred in part by the expression of the ABCG2 transporter protein, whereas Rhodamine dye exclusion is mediated by ABCB1/P-gp protein (15). Previous data have shown no correlation between the expression of ABCB1/P-gp in soft tissue sarcomas and tumor grade, and not surprisingly, Rho^{low} cells failed to initiate tumor formation (40, 41). To date, the role of ABCG2 transporter protein in soft tissue sarcomas has yet to be elucidated. However, it should be noted that *Bcrp1/Mdr1a/b* triple knockout mice still exhibit SP and it is likely that multiple transporters are involved in this process (42).

Further characterizations of the SP cells, such as determining differential potential and niche requirements, and the study of critical signaling pathways required for self-renewal are all studies that will bring this work closer to clinical applications.

Our studies show for the first time the existence of SP cells in primary mesenchymal tumors. Furthermore, we found that these cells are enriched for TICs. There seems to be a direct correlation between the number of SP cells present in a tumor and the aggressiveness of the tumor. Targeting this SP has the potential to be developed into an effective treatment modality for mesenchymal tumors.

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