Side Population in Human Lung Cancer Cell Lines and Tumors Is Enriched with Stem-like Cancer Cells

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Abstract

Stem cells have been isolated by their ability to efflux Hoechst 33342 dye and are referred to as the "side population" (SP). In this study, we used flow cytometry and Hoechst 33342 dye efflux assay to isolate and characterize SP cells from six human lung cancer cell lines (H460, H23, HTB-58, A549, H441, and H2170). Nonobese diabetic/severe combined immunodeficiency xenograft experiments showed that SP cells were enriched in tumor-initiating capability compared with non-SP cells. Matrigel invasion assay showed that SP cells also have higher potential for invasiveness. Further characterization of this SP phenotype revealed several stem cell properties. We found evidence for repopulating ability by SP to regenerate a population resembling the original population. SP displayed elevated expression of ABCG2 as well as other ATP-binding cassette transporters and showed resistance to multiple chemotherapeutic drugs. Human telomerase reverse transcriptase expression was higher in the SP, suggesting that this fraction may represent a reservoir with unlimited proliferative potential for generating cancer cells. mRNA levels of minichromosome maintenance (MCM) 7, a member of the MCM family of proteins critical to the DNA replication complex, were lower in SP cells, suggesting that a majority of the SP fraction was in the G0 quiescent state. Sixteen clinical lung cancer samples also displayed a smaller but persistent SP population. These findings indicate that SP is an enriched source of tumor-initiating cells with stem cell properties and may be an important target for effective therapy and a useful tool to investigate the tumorigenic process. [Cancer Res 2007;67(10):4827–33]

Introduction

Lung cancer is the most common cause of cancer death in the world today (1). Currently, lung cancer survival is poor with only 15% of patients surviving 5 years after diagnosis. Although new chemotherapy agents and radiotherapy have improved survival and quality of life of patients, the overall effect in the last decade has been mainly on palliation rather than reduction in mortality.

Evidence is accumulating that solid tumors, such as brain and breast cancer, contain a minor population of "cancer stem cells" that have high repopulation capacity (2, 3), a model that is already well established in hematopoietic malignancy (4). In vitro data have shown that only 1 in 1,000 to 5,000 lung cancer cells forms colonies in soft agar assay, indicating that not every lung cancer cell is capable of tumor initiation (5). Recent data from a mouse model reinforce the notion that lung adenocarcinoma arises from stem cells in the terminal bronchioles (6). Hence, it is likely that human lung cancers have rare stem-like cancer-initiating cells within the tumor.

Previous studies have shown that adult stem cells can be identified by a side population (SP) phenotype. The SP, first described by Goodell et al. (7), is a small subpopulation of cells with enriched stem cell activity that shows a distinct "low" Hoechst 33342 dye staining pattern. Later studies attributed this phenotype to expression of ABCG2, an ATP-binding cassette (ABC) transporter (8). Concurrent studies have shown SP cells in human cancers of different origins, including acute myelogenous leukemia, neuroblastoma, and glioma (9–12). These studies have suggested that the SP may be a source of cancer stem cells. If this is also the case in human lung cancer, it may be an important target for effective therapy for this disease.

The present study was undertaken to identify the SP in established human lung cancer cells maintained in long-term culture. We hypothesize that these cells represent an enriched fraction of tumor-initiating cells. Here, we report the presence of this small, distinct population in lung cancer cell lines that shows higher tumorigenicity in vivo and invasion in vitro compared with non-SP cells. They also exhibit several cancer stem cell properties, including high telomerase activity, quiescence, regeneration of both SP and non-SP, presence of ABC transporters, and multidrug resistance (MDR). Furthermore, a similar SP population was also found in 16 clinical lung cancer samples, raising important therapeutic implications.

Materials and Methods

Cell lines and clinical samples. Human tumor cell lines A549, H23, H460, HTB-58, H2170, and H441 were obtained from the American Type Culture Collection (ATCC) and maintained in culture medium recommended by ATCC. All media were supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS; Invitrogen-Life Technologies). All cell lines were incubated in a humidified incubator at 37°C supplied with 5% carbon dioxide. Cells were routinely maintained in 75 cm2 tissue culture flasks (BD Biosciences Discovery Labware) and harvested by 0.25% trypsin (Invitrogen-Life Technologies) treatment when they were in logarithmic phase of growth for SP analysis. Sixteen non–small cell lung cancers were obtained from patients undergoing surgical resection after informed consent. Briefly, fresh tumor samples obtained within 60 min of surgery were rinsed, mechanically minced, and digested for 4 h at 37°C in a shaking incubator with 0.1 Wünsch units/mL collagenase (Roche Diagnostics) in DMEM. The digest was further disaggregated through an 18.5-gauge needle and sieved through a 100-μm cell strainer to obtain single cell suspension. The single cell suspension was layered onto a 70% and 40%
Percoll gradient (Amersham Biosciences) and centrifuged for 20 min at 25,000 rpm, room temperature, and with no brake. Epithelial cells from the 70%/40% interface were then collected for SP analysis. Cell viability was determined by trypan blue exclusion.

**SP analysis.** The cell suspensions were labeled with Hoechst 33342 dye (Molecular Probes-Invitrogen) using the methods described by Goodell et al. (7) with modifications. Briefly, cells were resuspended at 1 × 10^6/mL in prewarmed DMEM (Invitrogen-Life Technologies) with 2% FCS (Invitrogen-Life Technologies) and 10 mmol/L HEPES buffer (Invitrogen-Life Technologies). Hoechst 33342 dye was added at a final concentration of 5 μg/mL in the presence or absence of reserpine (50 μmol/L; Sigma) and the cells were incubated at 37°C for 90 min with intermittent shaking. At the end of the incubation, the cells were washed with ice-cold HBSS (Invitrogen-Life Technologies) with 2% FCS and 10 mmol/L HEPES, centrifuged down at 4°C, and resuspended in ice-cold HBSS containing 2% FCS and 10 mmol/L HEPES. Propidium iodide (Molecular Probes-Invitrogen) at a final concentration of 2 μg/mL was added to the cells to gate viable cells. The cells were filtered through a 40-μm cell strainer to obtain single cell suspension before sorting. Analyses and sorting were done on a FACSVantage SE (Becton Dickinson). The Hoechst 33342 dye was excited at 357 nm and its fluorescence was dual-wavelength analyzed (blue, 402–446 nm; red, 650–670 nm).

**Tumor cell implantation experiments.** In vivo experiments were done in accordance with the institutional guidelines for the use of laboratory animals. SP and non-SP cells from H460 A549 and H441 were s.c. injected into nudeobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. For the limiting dilution assay, groups of mice were inoculated with SP cells at 1 × 10^3, 5 × 10^3, 5 × 10^4, and 1 × 10^5 or non-SP cells at 1 × 10^3, 5 × 10^3, and 5 × 10^4 (three to four mice per group). Tumor growth was monitored every 2 days after second week of inoculation. The mice were sacrificed at day 60 or when the tumors grow to a maximum of 1,000 mm^3. Tumor volume was calculated by the formula 0.52 × length × width^2. Fold difference in tumorigenicity was calculated by the following formula: (minimum number of non-SP cells needed to generate a tumor) / (minimum number of SP cells needed to generate a tumor). The tumors were surgically removed and digested in 0.1% Wünsch units/mL collagenase according to the manufacturer’s instructions before reanalysis by the Hoechst 33342 dye efflux assay as described above.

**Invasion assay.** Cellular potential for invasiveness of SP and non-SP cells was determined using six-well Matrigel invasion chambers (BD Biosciences Discovery Labware). Cells were seeded into upper inserts at 2 × 10^5 per insert in serum-free DMEM. Outer wells were filled with DMEM containing 5% FBS as chemotractant. Cells were incubated at 37°C with 5% carbon dioxide for 48 h, and then noninvading cells were removed by swabbing top layer of Matrigel with Q-tip. Membrane containing invading cells was stained with hematoxylin for 3 min, washed, and mounted on slides. The entire membrane with invading cells was counted under light microscope at 40× objective.

**RNA extraction and real-time PCR analysis.** Cells were harvested and total RNA was extracted using the RNeasy Micro kit (Qiagen). Total RNA was treated with DNase I (Invitrogen) and subsequently reverse transcribed using random hexamers and SuperScript II reverse transcriptase enzyme (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was done with SYBR Green Real-Time Core Reagents (Applied Biosystems) according to the manufacturer’s instructions on the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Primers were designed to generate a PCR product of 200 bp. Each 15 μL PCR contained 1.5 μL diluted cDNA (24 ng starting total RNA). Thermal cycling conditions were 50°C for 2 min and 95°C for 5 min followed by 40 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C. Levels of expression were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene.

**Drug sensitivity assay.** Fluorescence-activated cell sorting (FACS)-sorted cells were counted by the trypan blue assay for viability and seeded in 96-well plate format with appropriate growth medium at 100 μL per well. After 24 h of recovery, chemotherapeutic drugs (British Columbia Cancer Agency Pharmacy) were added at the IC_{50} concentration for each unsorted cell line (see Supplementary Table S1) and incubated for another 24 h. Sensitivity was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) cell proliferation assay [CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS), Promega] according to the manufacturer’s instruction. Briefly, 20 μL of substrate solution were added to cells and incubated for 2 h at 37°C. Absorbance was measured at 490 nm for each well using a microplate reader (Dynex Technology). Drug resistance was represented as % viability calculated using the following formula: (absorbance of treated cells) / (absorbance of untreated cells) × 100.

**Multiple staining analysis.** Cells were first stained with the Hoechst 33342 dye for 90 min as described above. Hoechst-stained cells were centrifuged and resuspended in HBFS (2% FCS + 0.05% NaN_3 + 5% human serum at a concentration of 5 × 10^6 to 7 × 10^7 per mL). Cells were stained with CD34-allophycocyanin (APC; BD Biosciences) and nestin-phycerythrin (PE; Cedarlane Labs) antibodies or with CD24-PE (BD Biosciences) and CD44-APC (BD Biosciences) antibodies at 10 μL per 1 × 10^5 cells. After incubation in the dark for 30 min on ice, 1 mL HBFS was added to each tube and centrifuged. Cells were resuspended in 300 μL HBFS with 2 μg/mL propidium iodide and analyzed using FACS. Staining profile for each marker was constructed within the SP and non-SP gates separately and compared.

**Statistical analysis.** Data were presented as the mean ± SD. To assess statistical significance of differences, an unpaired t test (GraphPad Software, Inc.) was done. P values <0.05 were considered significant as indicated by asterisks.

**Results**

SP phenotype is observed in human cancer cell lines. We examined the existence of SPs in six human lung cancer cell lines (see Materials and Methods) by staining them with Hoechst 33342 dye to generate a Hoechst blue-red profile. As a control, reserpine was added to block the activity of Hoechst 33342 transporter, and the SP gate was defined as the diminished region in the presence of reserpine. The profile of H460 was shown as an example (Fig. 1A). All the lung cancer cell lines contained a distinct fraction of SP cells, ranging from 1.5% (H23) to 6.1% (H441) of gated cells, which decreased significantly in the presence of reserpine (Fig. 1B).

**SP regenerates both SP and non-SP.** To compare the repopulation ability of lung cancer SP cells with non-SP cells, we cultured the sorted SP and non-SP cells separately under the same culture condition for 2 weeks before they were restained with Hoechst 33342 dye and reanalyzed. Both fractions were viable in culture, but the SP cells generated both a SP and a non-SP with a fraction size comparable with the original population, whereas the non-SP cells produced mainly non-SP cells (Fig. 1C and D).

**SP cells are more tumorigenic in vivo.** To test whether SP cells are enriched for tumorigenic cells, various numbers of SP and non-SP cells from H460, A549, and H441, cell lines known to give rise to tumors in vivo, were s.c. injected into mice and monitored for tumor development. As shown in Table 1 and Fig. 2A, H460 non-SP cells give rise to new tumors at 1 × 10^5 in only one of four mice tested. However, SP cells could form a tumor when only 5 × 10^4 cells (three of three animals) were inoculated, suggesting that H460 SP was enriched for tumor-initiating cells by at least 2-fold. This enrichment-fold is likely an underestimation because, at the same injection dose (1 × 10^7 cells), the tumor generated by the SP (1,350 mm^3) is 15-fold larger in volume than that of the non-SP (88 mm^3). For A549, SP cells gave rise to tumors with as little as 1 × 10^3 cells (two of four animals), whereas at least 5 × 10^5 non-SP cells were needed to form a tumor (three of four animals). Hence, A549 SP was significantly enriched for tumorigenic cells by 50-fold. H441 SP cells generated a tumor with 5 × 10^5 cells compared with...
at least $5 \times 10^4$ needed for non-SP to form a tumor, showing that H441 SP was at least 10-fold enriched in tumorigenicity. Reanalysis of SP-derived tumors by flow cytometry showed that, similar to SP cultured in vitro, SP cells under in vivo conditions also have the capacity to regenerate the SP and non-SP fractions (Fig. 2B).

**SP cells display increased invasiveness.** To investigate possible differences in invasiveness between SP and non-SP, an in vitro Matrigel invasion assay was done on sorted cells of each cell line. Figure 2C shows that SP cells of H460, A549, HTB-58, H441, and H2170 are all significantly more invasive than the non-SP cells.

**ABC transporters are up-regulated in SP cells.** Expression of ABC transporters has been shown in primitive cells and associated with its capacity to export a broad range of cytotoxic drugs (reviewed in ref. 13). In particular, ABCG2 has been implicated in the high Hoechst 33342 dye efflux capacity that marks the SP phenotype. Using a real-time reverse transcription-PCR (RT-PCR) assay, we determined the relative mRNA expression level of human ABC transporters in the SP and non-SP of lung cancer cell lines. Twelve ABC transporters were studied, including the four major drug transporters (ABCA2, MDR1, MRP1, and ABCG2) and related subfamily members (MRP2-MRP9). Consistent with previous reports, ABCG2 was elevated in the SP of all cell lines (Fig. 3A). In addition, the SP fraction expressed other drug transporters at a significantly higher level than the non-SP in H460 (ABC2, MDR1, and MRP1), A549 (ABCA2 and MRP1), HTB-58 (ABCA2, MDR1, and MRP1), H441 (MDR1), and H2170 (MDR1 and MRP1; Fig. 3B–D). Several related subfamily members were also found in higher levels in the SP (Supplementary Fig. S1).

**SP cells show higher resistance to chemotherapeutic drugs.** Because drug resistance was a common characteristic of normal

<table>
<thead>
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<th>Table 1. SP is enriched with tumorigenic cells</th>
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<td>Cell numbers for injection (tumor volume mm³)*</td>
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<tr>
<td>1 $\times$ 10³</td>
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<tr>
<td>----------------</td>
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<tr>
<td>H460 SP</td>
</tr>
<tr>
<td>Non-SP</td>
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<tr>
<td>Non-SP</td>
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<tr>
<td>H441 SP</td>
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<td>Non-SP</td>
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*Formula used for tumor measurements: 0.52 $\times$ length $\times$ (width)².

Figure 1. SP cells were present in lung cancer cell lines and exhibit repopulating activity. A, H460 cells were stained with Hoechst 33342 dye in the presence (right) or absence (left) of 50 μmol/L reserpine and analyzed by flow cytometry. The SP, which disappears in the presence of reserpine, was gated and shown as a percentage of the whole viable cell population. Square, portion of non-SP that was gated for isolation. B, size of the SP gate with or without addition of reserpine in other lung cancer cell lines. C, SP and non-SP cells sorted from H460 were cultured for 2 wks before restaining with Hoechst dye and reanalyzed. The SP (left) generated both a significant SP and non-SP fraction comparable with the original sort, whereas the non-SP (right) was mostly non-SP. D, percentages of SP regenerated from the SP and non-SP fraction for other lung cancer cell lines. *, $P < 0.05$, t test, statistical significance.
Figure 2. SP cells were more tumorigenic and invasive. 

A, representative s.c. tumors due to injection of H460, A549, and H441 SP cells compared with non-SP injection.

B, reanalysis of SP-derived tumor by the Hoechst dye efflux assay. 

C, invasiveness as measured by the Matrigel assay. SP or non-SP cells (4 × 10^5) were seeded and incubated for 72 h. Columns, number of cells invaded across the membrane. *, P < 0.05, t test, statistical significance.
and cancer stem cells, we tested whether the SP show heightened resistance to drugs commonly used in chemotherapy. We did sensitivity assays of seven chemotherapeutic drugs on SP and non-SP cells of H460, HTB-58, H2170, and H441 (both populations of these cell lines recovered in the first 24 h to yield reliable results). Table 2 shows that the SP of all cell lines exhibited higher resistance to chemotherapeutic drugs than non-SP. In particular, SP was resistant to all seven drugs tested for H460; to cisplatin and etoposide for HTB-58; to etoposide, gemcitabine, doxorubicin, and daunorubicin for H2170; and to etoposide, vinorelbine, and docetaxel for H441.

Human telomerase reverse transcriptase level is elevated in SP cells. To elucidate possible differences in telomerase expression between SP and non-SP, mRNA levels of human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase and limiting factor for telomerase activity, were measured and compared. As shown in Fig. 3E, hTERT expression was higher in SP cells for all cell lines tested.

Minichromosome maintenance 7 expression is lower in the SP fraction. Minichromosome maintenance (MCM) proteins are essential components of the replication helicase complex. They are useful markers that reflect the cell cycle state (14). We report here that the SP showed lower mRNA expression levels of MCM7, a member of the MCM family and a proliferation marker, in all cell lines tested (Fig. 3F).

Staining of SP cells for other putative stem cell markers. To elucidate on the possible association of other putative stem cell markers to the SP phenotype, cell lines stained by the Hoechst 33342 dye were additionally stained for CD24, CD34, CD44, and nestin. We found no significant difference between the SP and non-SP for each individual marker (Supplementary Fig. S2; Supplementary Table S2).

SP is present in clinical lung cancer samples. To see if clinical samples also contain the SP, we stained 16 surgical resections from lung cancer patients with Hoechst 33342 dye for FACS analysis. As shown in Fig. 4A and B, all samples tested showed a small SP (0.03–1.12%), showing the presence of this population in clinical lung cancer similar to lung cancer cell lines.

Discussion

Our present study is in keeping with literature supporting the existence of a SP, the fraction enriched for stem cells in long-term cancer cell culture. We showed that the SP could be reliably detected under the experimental conditions used. When injected into NOD/SCID mice, SP cells were found to be more tumorigenic than non-SP, which forms the majority of cells, thus indicating a significant enrichment of tumor-initiating cells in this small subpopulation. This is potentially important because effective curative therapy most likely depends on successful eradication of...
Table 2. SP shows high resistance to chemotherapeutic drugs

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<th></th>
<th>Cisplatin</th>
<th>Etoposide</th>
<th>Gemcitabine</th>
<th>Vinorelbine</th>
<th>Docetaxel</th>
<th>Doxorubicin</th>
<th>Daunorubicin</th>
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<td>H460 SP</td>
<td>42.0 ± 4.9</td>
<td>67.3 ± 3.8</td>
<td>33.9 ± 7.4</td>
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<td>49.4 ± 3.7</td>
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<td>H460 non-SP</td>
<td>30.9 ± 7.2</td>
<td>59.2 ± 3.5</td>
<td>26.9 ± 4.2</td>
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<td>HTB-58 non-SP</td>
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<td>H2170 SP</td>
<td>33.3 ± 3.1</td>
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<td>50.4 ± 3.7</td>
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<td>20.2 ± 0.7</td>
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<td>H441 SP</td>
<td>52.3 ± 3.9</td>
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NOTE: Values expressed as mean ± SD, done in triplicate in three independent experiments. Resistance quantified as percentage viability after drug exposure for 24 h. Statistical significance (P < 0.05) indicated by asterisks.

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these cancer-initiating cells. As observed by other investigators (9, 10), we also found both in vitro and in vivo evidence for the SP to regenerate a population of cells composed of both SP and non-SP, resembling the original unsorted population, thus showing repopulating capacities similar to stem cells. Moreover, our finding that lung cancer SP cells were more invasive than non-SP suggests that “stemness” may be related to invasiveness. Likely, there exists a population of stem-like cells within a lung tumor that is involved in the initiation of invasion.

Consistent with studies that show ABCG2 to be a molecular determinant of the SP phenotype (8), expression of ABCG2 mRNA was markedly higher in SP for all lung cancer cell lines analyzed. Interestingly, our results also revealed that the SP had elevated levels of other members of the ABC transporter family, including ABCA2, MDR1, and MRP1 (and related subfamily members MRP2 to MRP9 that are potential drug pumps), which are known to export different chemotherapeutic drugs and associate with drug resistance (13). Given that stem cells often display higher tolerance to cytotoxins (15), it is reasonable that SP cells in lung cancer also turn on several MDR genes as protective mechanisms. In support of this, we found that SP cells showed increased resistance to multiple chemotherapeutic drugs, several of which, notably cisplatin, gemcitabine, and vinorelbine, are commonly used as first-line therapy for lung cancer. Because different ABC transporters show overlapping yet different substrate specificity, the combination of these likely accounts for the range of drug resistance observed in the SP.

In this study, we found that expression levels of hTERT were elevated in SP cells from all cell lines tested. This is consistent with the work of Alvi et al. (16) that showed elevated hTERT level in the SP of normal mammary epithelium. In lung cancer, telomerase is expressed early in the multistage process and has been implicated...
in malignant transformation and tumor invasion. Furthermore, telomerase is a crucial marker of cellular immortalization in cancers (17). According to the cancer stem cell model, cancers likely have a subpopulation with indefinite repopulation potential. Thus, with its increased hTERT expression, the lung cancer SP may represent such a reservoir for generating cancer cells, driving cancer cell immortalization and disease progression. As proposed by previous studies, this telomerase expression in cancer stem cells may be inherited from their normal stem cell counterpart and is progressively lost during differentiation and maturation (18, 19).

MCM7 is an essential component of the replication helicase complex required for DNA replication. Its expression is required during the cell cycle, but in quiescent cells (G₀) it is found to be absent (14). Hence, it is a useful biomarker for proliferation. Here, we report that MCM7 expression was lower in the SP fraction, suggesting that SP cells are mainly outside of the active cell cycle. This is consistent with the concept that stem cells are mostly in the quiescent state (15).

In conclusion, our studies showed that it is possible to define and isolate an enriched tumor-initiating population in lung cancer using the SP phenotype. The cell lines investigated in this study contain SP cells that are significantly enriched for tumorigenicity and invasiveness. They also possess stem cell properties of MDR, high telomerase activity, repopulating capacity, and quiescence. Although the SP does not necessarily represent 100% pure cancer stem cells or all of the malignant stem cells from the whole population, it is a significant enrichment of these rare cells responsible for initiating and maintaining cancer. Because other potential stem cell markers, including CD23, CD34, CD44, and nestin, did not associate with Hoechst-dim cells, they cannot replace the Hoechst 33342 efflux assay in isolating lung cancer stem-like cells. Thus, the distinct SP phenotype currently provides an attractive testing model for studying lung cancer–initiating cell biology.

We report the existence of a similar small SP fraction, as defined by the Hoechst 33342 dye efflux assay, in 16 human clinical lung cancer samples. We propose that these SP cells also exhibit characteristics of a tumor-initiating, cancer stem cell phenotype. The presence of such a population with both high tumorigenic potential and drug resistance can have important clinical implications in lung cancer treatment. These rare cells have the potential to survive conventional chemotherapy and regenerate a cancer population, leading to relapse. Hence, the SP may represent both a useful predictor of treatment response and target for effective treatment. Future work will extend this characterization from human lung cancer cell lines into clinical specimens, potentially identifying important targets for therapy.

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References
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