Cancer Cells Cyclically Lose and Regain Drug-Resistant Highly Tumorigenic Features Characteristic of a Cancer Stem-like Phenotype

Kaijie He, Tong Xu, and Amir Goldkorn

Abstract

Drug resistance and brisk tumor initiation have traditionally been viewed as preexisting phenotypes present in small subpopulations of neoplastic cells sometimes termed cancer stem cells. However, recent work in cancer cell lines has shown that drug-resistant tumor-initiating features can emerge de novo within fractionated subpopulations of cells initially lacking these phenotypes. In the present study, we asked whether such phenotypic plasticity exists broadly in unperturbed cancer cell lines and tumor xenografts growing spontaneously without interventions such as drug selection or fractionation into subpopulations used in prior studies. To address this question, we used side population (SP) analysis combined with fluorescence labeling to identify a drug-resistant highly tumorigenic subpopulation and to track and analyze its interaction with the larger phenotypically negative population over time. Remarkably, we observed that SP size fluctuated in a cyclical manner: first contracting via differentiation into the non-SP (NSP) and then reexpanding via simultaneous direct conversion of numerous NSP cells back to the SP phenotype both in culture and in tumor xenografts. These findings show for the first time that adaptive, cancer-promoting traits such as drug resistance and brisk tumor initiation arise not only as solitary events under selective pressures but also as highly orchestrated transitions occurring concurrently in large numbers of cells even without specifically induced drug selection, ectopic gene expression, or fractionation into subpopulations. This high level of coordinated phenotypic plasticity bears consideration when using cancer cell lines as experimental models and may have significant implications for therapeutic efforts targeting cancer stem cells, which are marked by a drug-resistant tumor-initiating phenotype. Mol Cancer Ther; 10(6); 938–48. ©2011 AACR.

Introduction

The phenomena of drug resistance and tumor metastasis have long been recognized as central challenges in cancer therapy (1, 2). Traditionally, these properties have been conceptualized as preexisting molecular phenotypes randomly present in a small subset of cancer cells, sometimes termed cancer stem cells, that under the right environmental selection pressures (e.g., drug treatment or a new tumor site) would be favored to expand (3, 4). To study these adaptive responses, a variety of immortalized and transformed cell lines have been used; although offering only an approximation of in vivo cancer behavior, these models have allowed for serial labeling, tracking, and characterization of phenotypically distinct subpopulations over time. In this manner, small subpopulations of cells marked by drug resistance and vigorous tumor formation have been repeatedly identified and expanded (5–8).

Recently, the established unidirectional model of a unique, preexisting population selected to expand has been called into question by a number of high-impact studies: In several reports, cancer cells lacking the putative markers of drug resistance and brisk tumorigenicity were nonetheless capable of tumor formation under certain permissive conditions (9–11). In 2 recent reports, cells lacking drug-resistant and tumor-initiating features were clonally isolated and shown to be capable of reconstituting heterogeneous populations comprised both of cells with drug-resistant and tumorigenic features and of cells lacking those phenotypes (12, 13). These findings have raised the intriguing possibility that a preexisting seed population may not be necessary for the emergence of tumor-forming or drug-resistant phenotypes; rather, a subpopulation with these properties can arise de novo under certain conditions.
Given these observations, we wondered whether re-emergence of a drug-resistant highly tumorigenic phenotype could be achieved only under selective growth conditions or through isolation from other subpopulations as done in previous reports, or whether this was a more universal and spontaneous phenomenon. Specifically, could phenotypic plasticity be readily observed even in cell lines propagated under standard conditions without extrinsic selective pressures and without separation into constituent subpopulations? Moreover, did such plasticity represent a clonal selection of one phenotype from the other over time, or did it represent a real-time conversion occurring rapidly in many cells at once?

To investigate this question, we analyzed cancer cell lines in vitro and in vivo over time by using flow cytometry with Hoechst dye exclusion, a commonly used method that yields a side population (SP) of cells with drug-resistant highly tumorigenic properties in tumors and cell lines (14–18). Remarkably, when we coupled SP analysis with green fluorescent protein (GFP) labeling, we observed a dynamic 2-way equilibrium between the SP and nonside (NSP) subpopulations both in cell culture and in tumor xenografts. Specifically, the SP subpopulation first became depleted by differentiation into NSP cells and, subsequently, the SP subpopulation was reconstituted by direct conversion of numerous NSP cells simultaneously back to the SP phenotype; these transitions occurred spontaneously in the course of proliferation without exogenous selection pressures or separation into constituent subpopulations. Our findings show for the first time that intact cancer cell lines exhibit continuous, spontaneous plasticity whereby large numbers of cells lose and subsequently regain a drug-resistant highly tumorigenic phenotype in a cyclical manner. These observations suggest that adaptive traits that confer a survival advantage may be acquired by cancer cell populations not only through clonal selection of preexisting, solitary cells but also through an ongoing, highly orchestrated process of phenotypic interconversion occurring simultaneously in large numbers of cells to regenerate a cancer stem-like subpopulation.

Materials and Methods

Cell culture and lentiviral infections

Cancer cell lines were obtained from collaborators at the University of California, San Francisco, and the University of Southern California (see Acknowledgments) and were not reauthenticated before use in these experiments. Human bladder cancer cells (J82, RT4, UM-UC3), human breast cancer cells (MCF7), and rat glioma cells (C6) were maintained at 37 °C, 5% CO2 in Dulbecco’s Modified Eagle’s Medium (DMEM; MediaTech) supplemented with 10% of heat-inactivated FBS (Invitrogen), penicillin (100 units/mL; Invitrogen), and streptomycin (100 µg/mL; Invitrogen). Human prostate cancer cells (PC3, Du145, LNCaP, LNCaP-C4-2B) and human lung cancer cell (H441) were maintained in RPMI medium (supplemented with 10% FBS and antibiotics 100 units/mL penicillin and 100 µg/mL streptomycin).

GFP labeling of cells

Lentivirus was generated as previously described to deliver either GFP under a cytomegalovirus (CMV) promoter or a control empty vector (EV; ref. 19). One day before infection, 2 × 10⁴ J82 cells were seeded in 10-cm plates, and on the next morning, the medium was replaced by 3 mL virus supernatant plus 7 mL medium supplemented with 8 µg/mL polybrene. After 8 hours of incubation at 37 °C, the virus-containing medium was replaced with a fresh medium. Cells were observed for 48 hours to ensure more than 90% GFP expression before fluorescence-activated cell-sorting (FACS) and SP studies.

Flow cytometry

Hoechst staining and FACS were conducted as described previously (20). Briefly, adherent cancer cells (1 × 10⁶/mL) were trypsinized, counted, and resuspended in prewarmed 10% FBS DMEM. Hoechst 33342 (Sigma-Aldrich) was added at concentration of 5 µg/mL, incubated for 2 hours in 37 °C water bath, and gently inverted several times during the course of incubation. Parallel sample aliquots were prepared in the presence of 50 µmol/L verapamil (Sigma-Aldrich), an ATP-binding cassette transporter family inhibitor, at room temperature for 10 minutes before adding the Hoechst 33342 dye. Cells were centrifuged at 1,000 rpm for 5 minutes after incubation and resuspended in ice-cold DMEM. Propidium iodide (Sigma-Aldrich) was added to the cells at a final concentration of 2 µg/mL. Samples were incubated for at least 5 minutes on ice before FACS analysis (FACS Aria and FACSLSR-II; BD Biosciences; both equipped with UV lasers).

Drug resistance experiments

Cells were stained with Hoechst 33342 and fluorescence-activated cell-sorted into SP and NSP cells. Fractionated SP or NSP or unsorted WP (whole population) cells were seeded into 96-well plates at a concentration of 1 × 10⁴ cells per well in the presence of cisplatin (25 µmol/L) or docetaxel (0.4 mmol/L for J82 cells and 0.1 mmol/L for MCF7 cells, respectively). MTS assay was conducted after 24 hours according to manufacturer’s protocol (Promega).

Direct single-cell isolation

Single-cell suspension was made in DMEM and stained by Hoechst 33342 for SP analysis as described earlier. SP and NSP cells were sorted by FACS Aria directly into 96-well plates at the concentration of a single-cell per well (FACS 96-well sorting program), and single clone formation was confirmed visually on subsequent days.
Tumorigenicity assays

In vivo experiments were conducted under an approved protocol in accordance with the institutional guidelines for the use of laboratory animals. SP and NSP cells were suspended in 100 μL of media/Matrigel (1:1) and inoculated s.c. into the right flank of male severe combined immunodeficient mice (6–8 weeks old; NCI-Frederick). Time to onset of palpable tumor was recorded, and tumor diameter was measured twice weekly. Mice were sacrificed, and tumors were excised for wet weight measurement when the largest tumor diameters within each group exceeded 10 mm.

Analysis of excised tumors

In preparation for FACS, tumors were minced into small pieces (about 1 mm³) and immersed in digestion solution [DMEM/F12 (50:50) supplemented with DNAse I (1 μg/mL) and Liberase Blendzyme 3 (0.1–0.8 mg/mL)]. Cells were incubated at 37°C overnight, followed by trypsin incubation at 37°C for 5 minutes the next morning, and then trypsin neutralization with 10% FBS DMEM. Cell suspension was filtered first by a 100-μm strainer and then through a 40-μm strainer (BD Falcon) before FACS.

Colony formation assays

A total of 1,000 SP or NSP cells were seeded into 10-cm plates after FACS. As a control for the presence of Hoechst dye, 1,000 SP cells were treated with 50 μmol/L verapamil for 10 minutes and further stained by Hoechst 33342 for another 2 hours, and finally seeded into 10-cm plates. Cells were cultured at 37°C, 5% CO₂. Plates were washed 3 times with PBS, fixed with 100% methanol (room temperature, 15 minutes), and then stained for 1 hour at room temperature with 7 mL of Giemsastain (Sigma-Aldrich). After staining, cells were washed with water and air-dried overnight. The number of colonies with diameter of more than 1 mm was counted and photographed under a bright-field microscope (Nikon Eclipse TS100). All experiments were done in triplicate and repeated at least twice.

Quantitative reverse transcriptase-PCR assays

Total RNA was isolated from fluorescence-activated cell–sorted SP and NSP cells by using manufacturer’s RNA isolation protocol (RNA-Beckman-Coulter). RNA was reverse transcribed using a RETROscript kit (Ambion), and cDNA was then subjected to real-time PCR amplification with gene-specific primers and Quanta B-R Syber Green QPCR supermix (BioScience), with β-actin as a housekeeping gene loading control (primer sequences and conditions in Supplementary Table S1).

Statistical analysis

Statistical analysis was conducted in collaboration with USC/Norris Biostatistics Core. All experiments were conducted in triplicate, with error bars representing SD around the mean. Student’s t test was used to determine statistical significance when comparing mean values at one point in time (e.g., gene expression, tumor weights, SP percentage). Two-sided Wilcoxon matched-pairs signed-rank test was conducted by using the GraphPad Prism 5.0 Software to compare statistical significance of tumor growth over time.

Results

The SP is enriched for drug-resistant highly tumorigenic cells

We used Hoechst efflux and FACS analysis to identify SPs of cells with drug-resistant highly tumorigenic properties as previously reported in tumors and cell lines (14–18). Using this technique, we found SPs in a variety of cancer cell lines, including prostate, breast, lung, bladder, and glioma (Fig. 1A and Supplementary Fig. S1). Sorting of SP from NSP on a FACSArria flow cytometer (BD) yielded population purities of more than 91% and more than 97%, respectively, as confirmed on LSR-II cell analyzer (BD; Supplementary Fig. S2). The J82 and MCF7 cancer cell lines yielded SPs which constituted approximately 22.3% and 6.1%, respectively, of the overall populations and which diminished to 0.5% and 1.5%, respectively, after treatment with verapamil, which blocks the ABC transporter and prevents Hoechst efflux (Fig. 1A). To confirm that SP cells were enriched for drug resistance, J82 bladder cancer and MCF7 breast cancer cell lines were fractionated into SP and NSP and exposed to cisplatin and docetaxel, two chemotherapeutic agents commonly used in bladder cancer and breast cancer (Fig. 1B). For both cell lines, SP cells showed significantly greater survival than NSP or unfractionated WP; SP cells also had 2-fold mRNA expression of the ABCG2 transporter responsible for Hoechst and drug efflux (Supplementary Fig. S3).

SPs derived from MCF7 cells have been extensively characterized and previously shown to have high clonogenicity and tumorigenicity (14, 21); therefore, we next confirmed the same to be true of the J82 cancer cell line by using plating assays and SCID mouse inoculations. As expected, SP cells were significantly more clonogenic than NSP cells, generating approximately 3 times as many large colonies (>1 mm in diameter) at 5 weeks after seeding (Fig. 1C). To ensure that these clonogenic differences were not spuriously caused by retention of Hoechst dye in NSP cells and absence of dye in SP cells, we repeated the clonogenic assays in the presence of Hoechst dye + verapamil. Even when SP cells contained Hoechst dye similarly to NSP cells, they still were significantly more clonogenic than NSP cells (Fig. 1C). In vivo, SP cells formed tumors in all mice at all dilutions (5 × 10⁵, 1 × 10⁶, and 1 × 10⁷). In contrast, NSP cells formed tumors in only 1 of 3 mice inoculated with 1 × 10⁶ cells and in 2 of 3 mice inoculated with 1 × 10⁴ cells (Fig. 1D). Moreover, NSP-derived tumors formed only after a longer lag period and were smaller at excision than SP-derived tumors.
Thus, in summary, SP cells, relative to NSP cells, were found to be more drug-resistant, to form more colonies in vitro, and to form tumors more rapidly and efficiently in vivo, thus validating that SP cells indeed were enriched for a drug-resistant highly tumorigenic phenotype. In a further analysis, J82-derived SP cells also possessed higher mRNA levels of several genes associated with self-renewal (Supplementary Fig. S3), which was consistent with previous reports (14, 21, 22). At the same time, J82-derived and MCF7-derived subpopulations expressed very low or nondiscriminatory levels of CD133, ALDH1, and CD44+24low (Supplementary
Fig. S4), a finding that was not surprising, because these markers are known to be highly variable in different types of tumors and cell lines. In some cases, they correlate with high tumorigenicity and drug resistance, whereas in other cases they are uniformly expressed or totally absent (23, 24).

The NSP subpopulation generates SP and NSP subpopulations in vitro and in vivo

In the course of characterizing SP cells, we noted that NSP cells, though significantly less clonogenic and tumorigenic than SP cells, were nonetheless capable of forming some colonies in vitro and tumors in vivo (Fig. 1C and D). We wondered whether this delayed, inefficient capacity of NSP cells was predicated on reconstitution of a SP subpopulation, which, in turn, promoted colony and tumor formation. We tested this possibility by sorting J82 cells and several other cancer cell lines into SP and NSP subpopulations that were expanded and reanalyzed by FACS (Fig. 2A). J82 NSP cells reseeded in vitro reconstituted SP subpopulations that increased from undetectable at day 4 to ~6% at day 10 to ~12% at day 13 (Fig. 2B). Notably, we observed a similar phenomenon (Fig. 2B) in 3 other cancer cell lines previously shown to possess SP cells with drug-resistant highly tumorigenic properties (14, 18, 21, 25): MCF7 (breast cancer), C6 (rat glioma), and H441 (lung cancer). These NSP cells–derived subpopulations recapitulated the expected drug-resistant phenotypes, with SP showing significantly higher drug resistance than NSP (Fig. 2C). In vivo we inoculated SCID mice with equal numbers of J82 SP, NSP, or unsorted WP, then excised the xenograft tumors when they reached 10 mm in largest diameter, and analyzed the cells by FACS LSR-II. Notably, although NSP cells required a longer lag period before generating tumor xenografts (Fig. 1D), all the excised tumors, regardless of SP, NSP, or WP origin, contained similar (2%–3%) SP subpopulations (Fig. 2D). Thus, nondonor subpopulations from several different cancer cell types successfully reconstituted SP subpopulations over time in vitro and in vivo.

![Diagram](image-url)
Reconstitution of SP and NSP subpopulations from isolated NSP cells in vitro and in vivo could have conceivably resulted from imperfect sorting (97% purity, Supplementary Fig. S2) leading to unintentional inclusion of SP cells among the NSP population. To exclude this possible cross-contamination, we used FACS to sort single NSP cells directly into individual wells of 96-well plates and visually confirmed the formation of a single clonal population in each well (Fig. 3A). Significantly, NSP-derived single cells generated colonies in 78 of 600 wells (13%), a greater proportion than could have been caused by SP cross-contamination during FACS.Aria sorting, which has only 3% inaccuracy. A large majority (~75%) of the NSP-derived colonies, in turn, generated a significant SP subpopulation (defined as SP% > 1%; Fig. 3B), thus confirming that NSP cells indeed gave rise to SP cells and that this observation could not be explained by SP cross-contamination of NSP during sorting.

We extended the single-cell clone experiments in vivo to confirm that NSP-derived SP cells (termed secondary SP, or SP2°) reacquired the characteristic highly tumorigenic phenotype displayed by native SP cells; that is, the ability to efficiently form new tumors composed of heterogeneous cell subpopulations. To test this, NSP-derived clones were fluorescence-activated cell sorted into secondary SP and NSP subpopulations (SP2° and NSP2°) and inoculated into SCID mice. Although both SP2° cells and NSP2° cells were capable of forming tumors, SP2° cells were significantly more tumorigenic than NSP2° cells (Fig. 3C). Subsequent xenograft excision and FACS analysis (Fig. 3D) confirmed reconstitution of heterogeneous cell subpopulations in all tumors; both SP2° and NSP2° generated tumors with side and...
NSP subpopulations, and there was no significant difference in SP percentage between these groups. Collectively the single-cell clonal experiments confirmed both in vitro and in vivo that NSP cells could reconstitute SP subpopulations with a characteristic highly tumorigenic phenotype.

**The SP expands and contracts cyclically in the course of cell culture**

Having observed that NSP cells were capable of reconstituting SP + NSP populations in vitro and in vivo, we sought to characterize the day-by-day dynamics of these subpopulations growing in a native state (i.e., together in an intact cancer cell line). To accomplish this, we propagated unsorted J82 cells in cell culture and monitored daily the size of the SP, NSP, and WP. Unexpectedly, we found that within 1 day of seeding J82 cells into a new plate, the SP subpopulation became rapidly depleted from a baseline of approximately 20% down to approximately 0.5% of the total population (Fig. 4A). In subsequent days, the SP subpopulation experienced a gradual increase, punctuated by a sudden spike in SP subpopulation size on day 3 or 4 (Fig. 4A, open arrows). The SP subpopulation continued to increase, approaching its original baseline percentage (20%) as the overall culture neared confluence. At that point, the cells were passaged into new plates and the cycle was repeated. Interestingly, the sudden increase in SP percentage on day 3 or 4 was independent of the overall expansion rate of the cultured J82 cells; in fact, SP expansion at those inflection points (Fig. 4B, open arrow) was approximately 4- to 7-fold more rapid than the expansion of the NSP subpopulation or of the entire population as a whole (WP).

**SP cells arise through simultaneous direct conversion of numerous NSP cells**

We observed that the SP subpopulation experienced a sudden, marked expansion at approximately mid-passage, a finding that could be explained 1 of 2 ways: According to the traditional model, drug-resistant highly tumorigenic cells could arise only through expansion of preexisting cells, implying that the observed sudden SP expansion was caused by an explosive increase in SP cell proliferation. An alternative explanation for the sudden SP expansion was that SP cells were arising by conversion of cells from the NSP subpopulation rather than by proliferation of preexisting SP cells alone. To test this hypothesis, we generated a GFP-based model that enabled us to track the fates of the SP and NSP subpopulations (Fig. 5A). Briefly, J82 cells were infected with lentivirus expressing either EV or GFP. Both J82EV and J82EV were fluorescence-activated cell sorted into their respective SP and NSP subpopulations. These were recombined to create a J82 cell line consisting of 20% SP\(^\text{EV}\) and 80% NSP\(^\text{GFP}\). This hybrid cell line then could be analyzed daily by FACS for Hoechst dye efflux and GFP status.

When analyzed daily by FACS during in vitro culture, the reconstituted J82 cells experienced the same initial (days 1–2) depletion of SP cells observed previously, from ~22% down to 0.7% (Fig. 5B). During this early phase (days 1–2), the SP cells (red) migrated over to the NSP gate. Then, as the SP percentage gradually increased on days 3 to 6, there was a concomitant gradual increase in the GFP\(^+\) percentage (green cells) within the SP gate (Fig. 5B). At the day 6 inflection point, the sudden sharp increase in SP subpopulation was mirrored by an equally sharp increase in GFP\(^+\) percentage within the SP gate. These findings confirmed that the rapid reexpansion of the SP subpopulation was not caused by intrinsic self-renewal and proliferation of preexisting SP cells but rather by conversion of NSP cells into the SP phenotype. Notably, this phenomenon was confirmed (and any confounding role of GFP protein expression was ruled out) with a reverse experiment wherein 20% SP\(^\text{GFP}\) and
Figure 5. SP cells arise through the conversion of NSP cells. A, schematic of GFP experiments. FACS-fractionated SP^GFP cells were mixed with NSP^GFP cells in a 20:80 ratio at day 0; a total of 1 x 10^6 of the mixed cells were cultured in 6-well plates for in vitro analysis or inoculated into SCID mice for in vivo xenograft tumor analysis. B, in vitro analysis. Left, daily FACS plots showing the relative proportions of GFP^+ and GFP^− cells in the SP and NSP gates over time. Right, graphs plotting the relative size of SP and the GFP^+ proportion within the SP over time. C, in vivo analysis. Left, FACS plots showing the relative proportions of GFP^+ and GFP^− cells in the SP and NSP gates at 3 time points. Right, graphs plotting the relative size of SP and the GFP^+ proportion within the SP in xenograft tumors derived from the 20:80 SP^GFP/NSP^GFP J82 cancer cell line. D, illustrated model summarizing the direct interconversion observed between the drug-resistant highly tumorigenic phenotype (DRHT, SP) and the drug-sensitive low-tumorigenic phenotype (DSLT, NSP). This plasticity occurred cyclically in many cells at once, suggesting a possible adaptive response to environmental factors (italicized with question marks).
80% NSP<sup>EV</sup> were combined with similar results (Supplementary Fig. S5). Hence, these experiments provide the first direct evidence that the drug-resistant highly tumorigenic phenotype within an established cancer cell line is rapidly and simultaneously acquired by numerous cells previously lacking this phenotype.

The principle of phenotypic plasticity was borne out further in vivo. The hybrid J82 cell line consisting of 20% SP<sup>EV</sup> + 80% NSP<sup>GFP</sup> was inoculated into SCID mice, and the xenograft tumors were resected at 3 time points and analyzed by FACS for SP percentage and GFP percentage (tumor gating accuracy and persistence of GFP expression were confirmed in separate experiments; Supplementary Fig. S6). Interestingly, the proliferating tumors recapitulated the early, rapid, proliferative phase observed in vitro; that is, the SP percentage declined from 20% in the hybrid J82 preincubation down to approximately 1% to 3% in the xenograft tumors at subsequent time points (Fig. 5C). Consistent with this trend, FACS plots of resected tumors showed that the original SP<sup>EV</sup> (red) cells migrated over time to the NSP gate by day 69. At the same time, the GFP<sup>+</sup> percentage within the SP gate increased from 30% GFP<sup>+</sup> preincubation to approximately 90% GFP<sup>+</sup> in the xenograft-derived SP subpopulations, suggesting a progressive replacement of the original SP subpopulation expressing EV (SP<sup>EV</sup>; largely GFP<sup>+</sup>) by a new GFP<sup>+</sup> side population derived from the NSP<sup>GFP</sup> cells (Fig. 5C).

Hence, in tumor xenografts, as in vitro, drug-resistant highly tumorigenic SP subpopulation was replenished by conversion of cells from the nonside subpopulation. As illustrated in the summary model (Fig. 5D), the observed phenotypic interconversions were not only spontaneous but also cyclical and occurring simultaneously in large numbers of cells, a nonrandom pattern suggestive of a deliberate process, perhaps in response to some environmental factor or factors.

**Discussion**

We investigated whether emergence of drug-resistant highly tumorigenic cells could be observed in cancer cell lines propagated in culture and as tumor xenografts. Specifically, we asked whether phenotypic plasticity could be observed even in unfractionated, intact cell lines grown without specific selective pressures such as chemotherapy treatment. Moreover, could direct phenotypic conversion within groups of cells, rather than clonal selection over time, be documented as an adaptive mechanism used by these cell lines. To study these questions, we used established cancer cell lines that contained a Hoechst-effluxing SP characterized by drug resistance and high tumorigenicity (Fig. 1; refs. 5, 6, 8, 14, 18, 21, 25, 26). We recognize that cancer cell lines may not perfectly represent the biology of spontaneous in vivo tumors; however, tumor tissues and primary tumor–derived cultures do not lend themselves to serial labeling and analyses of phenotypically distinct subpopulations over time. In contrast, cell lines do allow this type of characterization and therefore have been used extensively to explore how drug-resistant highly tumorigenic phenotypes emerge (3, 7, 12, 13, 27). Cancer cell subpopulations possessing these properties have sometimes been referred to as cancer stem cells or tumor-initiating cells (28), because their typically high expression of self-renewal genes (also noted in our studies, Supplementary Fig. S3) conjures a stem-like, progenitor or hierarchical association. However, as reported previously (24) and noted in our own studies (Supplementary Fig. S4), particular markers can vary widely in different tumors and cell lines; therefore, for the purpose of these studies, we opted for terminology based strictly on functional, therapeutically relevant properties that were empirically shown in our SP/NSP experiments: drug resistance and high tumorigenicity.

As expected, fluorescence-activated cell–sorted SPs gave rise to entire heterogeneous populations (SP + NSP) in vitro and in vivo; however, NSPs from all of the cancer cell lines also were capable of reconstituting heterogeneity (SP + NSP) albeit less efficiently (Figs. 1 and 2), a finding that we validated with single-cell clone experiments (Fig. 3). These findings were consistent with a recent study using embryonic stem cells wherein NSP was shown to give rise to SP (29), as well as with recent observations in cancer models wherein cells lacking the canonical surface markers for a highly tumorigenic phenotype were nonetheless capable of tumor formation (9–11, 15, 16). A host of explanations have been proposed to account for the observed tumorigenicity of marker-negative cells: limited discrimination of cell surface markers, cross-contamination during sorting, and variability of mouse models used to gauge tumorigenicity (9–11, 16, 18). However, another plausible explanation shown in 2 recent articles (12, 13) and supported by our own findings is that drug-resistant highly tumorigenic subpopulations could arise directly from cells that initially lack these traits.

Having confirmed that fractionated subpopulations expanded in isolation could reconstitute phenotypic heterogeneity, next, we wished to better define the interaction of these subpopulations in an intact unfractionated cell line (J82) by tracking the SP and NSP cells in real time. Remarkably, we found that the side subpopulation size fluctuated cyclically with each passage in cell culture (Fig. 4). Fluctuations in SP size have been reported previously with exposure to radiation, hypoxia, or ectopic activation of specific signaling pathways and were attributed to intrinsic changes in SP proliferation in response to deliberate external stimuli (21, 30–33). In contrast, our experiments applied no external stimuli to the cultured cells; nevertheless, we unexpectedly observed cyclical SP fluctuations occurring spontaneously in 2 phases: First, the SP subpopulation contracted drastically after seeding, diminishing from approximately 20% to less than 1% in the first 2 days. There are 3 possible explanations for this: (i) SP cells died out in the first 2 days—this is unlikely, as no such
Phenomenon was observed in our prior experiments using fractionated SP cells. (ii) SP cells did not proliferate and therefore were relatively swamped out by the NSP cells—this also is unlikely because the overall population doubling time (~24 hours) could not possibly reduce the SP percentage 20-fold in 2 days. (iii) SP cells rapidly differentiated out into NSP cells—this explanation is most consistent with the classic drug-resistant highly tumorigenic phenotype and is in fact borne out by the subsequent GFP-labeling experiments, wherein GFP− cells were observed to migrate from the SP gate to the NSP gate in the first 2 days (Fig. 5).

The second phase in the cyclical fluctuation of SP size was even more striking: At every passage, as the cell culture passed mid-passage and began to approach confluence, the SP subpopulation underwent a rapid expansion (Fig. 4). When we GFP-labeled the NSP subpopulation (Fig. 5), we observed that the rapid SP expansion derived not from intrinsic proliferation of preexisting SP cells but rather from direct conversion of NSP cells to the SP phenotype, a phenomenon that was recapitulated in vivo when labeled cells were xenografted into SCID mice. Thus, both in vitro and in vivo, the SP subpopulation was replenished by conversion of cells from the NSP subpopulation.

The results of our experiments suggest a higher order of population-wide plasticity than previously suspected. Recent reports showed that drug-resistant tumor-initiating traits could arise de novo within fractionated phenotypically negative subpopulations expanded in isolation or under selective pressures, suggesting that solitary cells or small numbers of cells were capable of altering their phenotypes under these ectopically induced conditions (12, 13, 30, 31, 33, 34). Our current findings show for the first time that such plasticity is actually a robust phenomenon that can occur spontaneously in intact cell lines without the need for fractionation or selective pressures. Specifically, a drug-resistant highly tumorigenic phenotype is lost and subsequently regained by subpopulations of cells during passaging in culture, and similar phenotypic interconversion is also observed in tumor xenografts. Interestingly, work done in parallel to ours using a different cancer model resulted in similar observations reported contemporaneously with our own findings (35).

Importantly, although the observed phenotypic interconversion was spontaneous (no ectopic drugs or gene expression), it was not entirely random. Rather, it was a cyclical, highly orchestrated process occurring simultaneously in large numbers of cells, suggesting a real-time adaptive capability, perhaps in response to environmental factors such as availability of nutrients, oxygen, or space (36, 37). This type of rapid, population-wide adaptation constitutes an alternative to the traditional model wherein preexistent traits that confer a survival advantage are expanded in a blind, passive manner under selective pressures. Instead, entire populations of cancer cells may possess innate, programmed adaptive responses that are activated by real-time sensing and response to environmental conditions. According to this hypothetical dynamic model (Fig. 5D), when cancer cells are introduced into a new, permissive growth environment (e.g., culture dish or s.c. inoculation), the drug-resistant highly tumorigenic subpopulation rapidly differentiates into the bulk phenotypically negative population and thus effectively populates the new environment. Conversely, as that environment becomes saturated and less hospitable, perhaps through depletion of critical resources (nutrients, space, oxygen) or accumulation of toxins, a portion of phenotypically negative cells respond by reverting to a drug-resistant highly tumorigenic phenotype, which is more robust and better able to sustain the cancer population until a new permissive environment (i.e., new culture plate or metastatic site) is encountered and the cycle repeats. Indeed, this type of highly orchestrated direct conversion of phenotypically negative cells to a drug-resistant highly tumorigenic phenotype may underlie some of the changes in subpopulation size previously observed in response to deliberate manipulations (21, 30–34), as well as the observed capacity of phenotypically negative cells to form tumors (perhaps by first reconstituting a highly tumorigenic subpopulation in vivo).

The environmental signals and cellular mechanisms governing these phenotypic interconversions are the subject of intense investigation by our group and are as yet to be fully elucidated. At the same time, the very presence of this phenomenon has important experimental and clinical implications. Experimentally, cancer cell lines, so commonly used to study drug resistance and tumor formation, should be used with the recognition that phenotypic heterogeneity exists in subpopulations that are engaged in a continuous state of flux. Clinically, phenotypic plasticity, if confirmed in additional models, would have significant implications for cancer therapeutics. Current efforts to eradicate drug-resistant highly tumorigenic subpopulations as distinct entities may meet with limited success, because this phenotype may simply be replenished by the phenotypically negative population. Hence, it may ultimately prove more productive to conceptualize drug resistance and high tumorigenicity as adaptive phenotypes assumed by a subset of the cancer population rather than as discrete, static subpopulations of cancer stem cells. Elucidating the signals and mechanisms that govern this dynamic, ongoing plasticity may ultimately lead to more effective therapeutic strategies aimed at disrupting the adaptive capabilities of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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