Cancer Stem Cells and Aneuploid Populations within Developing Tumors Are the Major Determinants of Tumor Dormancy

Anjali P. Kusumbe and Sharmila A. Bapat

Abstract

Tumor formation involves substantial cell division and genetic instability, but the relationship between quiescent cancer stem cells (CSC) and dividing progenitors in these events is poorly understood. Likewise, the implication of aneuploid cells in solid tumors is uncertain. CSCs are postulated to contribute to tumor dormancy and present a formidable obstacle in limiting treatment outcomes for a majority of cancers, whereas the genetic heterogeneity conjured by aneuploid cells may influence tumor drug resistance. However, direct confirmation of these events remains forthcoming. In the present study, we addressed the identification of tumor dormancy in terms of isolation of therapy-refractory residual tumor cells from tumors that persist in a state of quiescence as label-retaining cells. The choices of label were PKH67/PKH26 dyes that irreversibly bind to the lipid bilayer on cell membranes and get equally partitioned among daughter cells subsequent to each cell division. Consequent characterization revealed that label-retaining cells encompass two different populations capable of remaining in a state of quiescence, i.e., stem-like cells and aneuploid cells. The former express a reversibility of quiescence through retention of functionality and also exhibit therapeutic refractoriness; the latter seem to be either quiescent or proliferation-arrested at steady-state. Subsequent to exposure to selective pressure of chemotherapy, a fraction of these cells may acquire the potential to proliferate in a drug-refractory manner and acquire stem-like characteristics. Collectively, the findings of the present study reveal that tumor-derived CSCs and aneuploid populations contribute to drug resistance and tumor dormancy in cancer progression.

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Introduction

Tumor dormancy represents a subclinical equilibrium achieved between host immunity and quiescent residual tumor cells (1), that can extend for up to decades after treatment followed by disease relapse (2, 3). Also interpreted as being the minimal residual disease, this state remains a major hurdle in achieving complete remission (4, 5). The exact identity and functional characterization of cells that contribute to tumor dormancy, i.e., the cellular components of minimal residual disease lesions remain poorly understood. In recent years, the association of tumor dormancy with cancer stem cells (CSC) has been extensively speculated following their identification in diverse cancers including breast, brain, ovary, prostate, etc. (6–9), based on their analogy with adult stem cells in terms of a high capacity for self-renewal and multi-lineage differentiation (10). A more primeval trait of stem cells in adult organs is quiescence, which is critical in tissue proliferation kinetics and maintaining tissue homeostasis (11). In an analogous manner, CSCs are speculated to remain quiescent; tumor dormancy is conjectured to correlate with this quiescent CSC fraction that is likely to persist posttherapy to drive disease relapse. Aneuploidy—described as a hallmark of cancer—is associated with proliferation defects and may impart selective, adaptive advantages under a specific permissive context to tumor cells (12, 13). This forms a strong case for aneuploid cells contributing to tumor dormancy. Unfortunately, a definitive identification of CSC and/or aneuploidy-related quiescence has remained elusive due to a paucity of experimental data.

Long-term label retention is widely used for the identification of stem cells (14–16). A standard label-retaining cell (LRC) assay exploits the slow-cycling nature of stem cells (the reason they retain their labels), whereas rapidly dividing, transit-amplifying (TA) progenitors generated through stem cell self-renewal (17), dilute their labels. LRCs thus represent experimentally marked slow-cycling stem cells. Although bromodeoxyuridine labeling of DNA is frequently used in LRC assays, recent studies show its cytotoxic nature (18). The use of membrane-labeling dyes such as PKH67/PKH26 that have a good correlation with bromodeoxyuridine incorporation and slow-cycling cells is suggested (19–21). These vital dyes consist of a fluorophore attached to an aliphatic carbon backbone that irreversibly binds to the lipid bilayer on cell membranes. Subsequent to each cell division, the label gets equally partitioned among daughter cells, resulting in the reduction of fluorescence intensity (22, 23). PKH labeling enables the monitoring of live cells and is useful when subsequent functional analysis of labeled cells is required (24). In the present study, we explored the application of PKH label retention/quenching as an effective readout for the identification of quiescent cells in tumors. Such an assessment revealed CSCs and aneuploidy as two contrasting nonproliferative states in tumors that can be coaxed into regeneration, thereby providing a novel understanding of the cellular determinants of tumor dormancy.

Materials and Methods

Cell lines, cell culture, and PKH labeling of cells. A4 cells used in the study were established earlier in our lab from malignant ascites of a patient diagnosed with grade 4 serous ovarian adenocarcinoma (10). Paclitaxel-resistant A4 cells (A4CR) were developed from parental A4 cells using standard procedures (25). Human tumor cell lines NT2, PA1, HL60, C6, U87, and T47D were obtained from the National Centre for Cell Science Repository and maintained as recommended. PKH labeling was performed as described earlier (24).

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

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Animal housing, generation of tumors, and paclitaxel treatment. Nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) mice were bred and maintained at the Experimental Animal Facility, National Centre for Cell Science; all procedures were done in accordance with institutional ethical animal committee clearances, laws, and policies. Animals were kept under sterile airflow conditions during the experiment. For subcutaneous tumor formation, either $4 \times 10^6$ unlabeled A4 cells (control group), or $4 \times 10^6$ PKH26 or PKH67-labeled A4 cells were injected s.c. into the thigh of 5- to 6-week-old female NOD/SCID mice. Drug treatment of mice was with 25 mg/kg of paclitaxel, whereas vehicle control mice were exposed to DMSO (26). Further details of animal experiments are provided in the Supplementary Methods.

**Tumor digestion for flow cytometry and analysis.** Tumors were finely minced and subjected to collagenase digestion (collagenase IA and collagenase XI—both 5 mg/mL in RPMI medium) to obtain single-cell suspensions. CD44/c-kit staining and Ki67 staining were performed using standard protocols. PKH-based sorting was performed on FACSAria (Becton Dickinson) for semiquantitative PCR, quantitative PCR, clonogenicity analyses. PKH intensity plots of labeled tumors yielded a continuous gradient of cells with high intensity similar to preinjected cells to cells devoid of label. PKHhi, PKHlo, and PKHneg fractions were identified, gated, and sorted from this continuous gradient for subsequent analyses. Data analysis was with FACSDiva software.

**Demarcation of euploid and aneuploid populations and distribution analysis of their cell cycle phases.** Propidium iodide (PI)-based DNA content analysis does not resolve G0 and G1 cell cycle phases, which is achieved through Hoechst (DNA content)-Pyronin Y (RNA content) staining. Briefly, cells were stained using Hoechst 33342 (5 μg/mL for 45 min, at 37°C in staining buffer); 1 μg/mL of Pyronin Y was added and cells were incubated for an additional 45 min prior to washing and flow cytometry. Although Hoechst-Pyronin Y staining was applied for the identification of quiescent diploid stem cells from normal organs; it was not applied to tumor-derived cells that had heterogeneous DNA content. Our analysis of tumors indicated that whereas this staining enabled the demarcation of quiescent (G0), euploid (P1), and quiescent (G0) aneuploid (P2) fractions, euploid S–G2/M and aneuploid G1 phases (P4) merge on the histogram. Another drawback is that S–G2/M phases are not resolved within the P5 fraction. PI staining however permits a lucid demarcation of S–G2/M phases of euploid and aneuploid fractions. Thus, towards complete cell cycle profiling, both methods were performed, analyzed independently, and further overlapped to identify the frequencies of individual phases in euploid as well as aneuploid populations.

**Semiquantitative and quantitative reverse transcription-PCR.** Semiquantitative and quantitative reverse transcription-PCR was performed under standard conditions as described earlier (24).

**Statistical analysis.** Unless mentioned otherwise, all experiments were carried out in triplicate; data are expressed as mean ± SEM of at least three independent experiments. The significance of difference in the mean values was determined using two-tailed Student’s t test. $P < 0.05$ was considered significant.

**Results**

Differential label retaining capacities suggestive of proliferation heterogeneity are expressed by cells within primary tumors and metastases. To visualize LRCs by flow cytometry, ovarian cancer cells (A4) were labeled with the membrane dye PKH67 and injected s.c. into NOD/SCID mice for tumor formation (Fig. 1A, horizontal column; Materials and Methods). Analyses of 3-week-old tumors ($n = 11$) for distribution of PKH intensity revealed a continuous gradient of cells with fluorochrome PKH67 retention ranging from high (PKHhi, equivalent to preinjected cells), low (PKHlo cells), to total label quenching (PKHneg, Fig. 1A, vertical column). Such profiles could be consistently identified in all tumors ($n = 25$) ranging from short-term (2 weeks) to fairly long-term (6 weeks). However, the cell distribution frequencies of each fraction varied between short-term and long-term tumors, with the size of the PKHhi fraction diminishing with tumor progression (Supplementary Fig. S1A); this could partly be an effect of increasing tumor mass with time. Ovarian cancer progression is characterized by the formation of malignant ascites and the development of secondary peritoneal tumors; the A4 cells model this pattern in immunocompromised mice (10). Our next attempt was to decipher label retention during tumor progression. PKH67-labeled A4 cells were injected i.p. in NOD/SCID mice; 40 days postinjection, multilayered spheroids, single cells within the ascites and secondary tumors, were collected, digested, and analyzed by flow cytometry and/or immunofluorescence. At all metastases-derived cells showed the three cell fractions differentiated on the basis of their label retention (Supplementary Fig. S1B). To further elucidate whether the PKH-based gradient identified in A4-derived tumors is cell type-specific or represents a general scheme during tumor formation, we analyzed 3-week-old tumors ($n = 3$) generated in NOD/SCID mouse models from PKH67-labeled C6 cells (a rat glioma–derived cell line). Consequently, the three fluorochrome-based cell subsets differentiated in A4 cells were also identified in C6-derived tumors (Supplementary Fig. S1C). Because label quenching is a direct function of fluorochrome dilution following each consecutive cell division, the profiles generated are strongly suggestive of the subsistence of proliferative heterogeneity within primary tumors and their metastases consisting of three distinct populations: (a) label-retaining PKHhi cells suggested to be slow-cycling/quiescent, (b) PKHlo cells that undergo partial label dilution indicative of limited divisions, and (c) PKHneg cells that undergo total dye quenching suggestive of consecutive, rapid divisions.

**Label-retaining PKHhi cells exhibited the highest self-renewal potential and stem cell marker expression.** To correlate between slow-cycling, proliferation, and self-renewal capabilities, the three freshly sorted PKH tumor cell fractions were subjected to colony formation assays in vitro and tumorigenicity in vivo. The clonogenic potential of PKHhi cells was significantly higher than that of PKHlo cells, whereas the PKHneg subset lacked colony formation potential (Fig. 1B, top). In vivo, the high tumorigenic nature of the PKHlo subset was clearly shown wherein only 5,000 cells were capable of initiating and developing tumors in all animals injected (Fig. 1B, bottom). The minimal threshold of tumor initiation for the PKHhi subset was $\sim 10,000$ cells; whereas PKHhi cells failed to display tumorigenic potential. These analyses indicated that the tumor-initiating capacity of the A4 population lies primarily within the PKHhi, and to a lesser extent, in the PKHlo subset. The three tumor cell subsets were also screened for expression of stem cell markers Oct4, Nestin, Nanog, and Bmi. Oct4 and Nestin expressions were exclusively associated with the PKHhi subset, whereas Nanog and Bmi were expressed in PKHlo and PKHhi subsets with expressions being higher in the former (Fig. 1C, top). The PKHhi subset lacked expression of all four markers. On studying the association of a recently described cell surface phenotype of ovarian CSCs (CD44+/c-kit+; ref. 27) with the three tumor cell subsets, these expressions were totally absent in the PKHlo subset, whereas 68% of PKHhi and the entire PKHlo subset were enriched for double-positive expression (Fig. 1C, bottom). We also probed the expression of the proliferation marker Ki67 (expressed in late G1, S, and G2/M phases of cell cycle) in these fractions. Approximately half (57%) of the PKHlo subset and 7% of PKHhi and 7.1% of PKHneg cells expressed Ki67 (Fig. 1D). In totality, our results suggest that the PKHhi subset represents label-retaining, slow-cycling stem-like cells; the PKHlo subset as a progenitor population (most...
likely TA progenitors); and the PKH\textsuperscript{neg} subset as the differentiated fraction within tumors (the latter may additionally harbor some host-derived cells that would not express the label).

The PKH\textsuperscript{lo} subset comprised of proliferation-arrested aneuploid cells and euploid progenitor cells. PI-based cell cycle analyses of 3-week-old tumor-derived cells further confirmed the proliferation kinetics of the three subsets and lent support to the above classification identified through functional and phenotypic expression analyses (Fig. 2A). Although the PKH\textsuperscript{hi} fraction seems to represent the immediate progeny of PKH\textsuperscript{hi} CSCs, a striking feature revealed from the PI profiles was that a major fraction of these cells expressed enhanced ploidy levels (DNA content 4N or more as compared with baseline A4 euploid DNA levels). Such aneuploid cells were also present in the PKH\textsuperscript{hi} population albeit to a lesser extent, but did not appear in the PKH\textsuperscript{neg} fraction. The absence of such cells prior to injection (Supplementary Fig. S2A) suggested the

Figure 1. Identification of LR-CSCs within the tumors. A, experimental design for PKH label based on in vivo detection of quiescent cells within tumors. Representative PKH intensity profiles of preinjected cells, 3- and 6-wk A4 tumors. B, clonogenic potential of tumor-derived PKH\textsuperscript{hi}, PKH\textsuperscript{lo}, and PKH\textsuperscript{neg} cells (top). Tumorigenicity of these fractions and unsorted A4 cells transplanted s.c. into NOD/SCID mice (bottom). C, semiquantitative PCR analyses (top) of stem cell markers in the three PKH cell populations; β-actin mRNA expression used as internal control. D, representative contour plots of flow cytometric analysis for expression of ovarian CSC markers c-kit/CD44 (bottom) and proliferation marker Ki67 in the PKH subsets.
association of in situ tumor development with genetic instability and rearrangements that could lead to the emergence of aneuploidy. These processes are identified as hallmarks of tumorigenesis (12); however, their existence was further affirmed through DNA content analysis of primary ovarian cancer samples (eight tumors and one malignant ascites) that harbored 1.87% to 6.74% (mean = 4.16 ± 1.54%) aneuploid cells (Supplementary Fig. S2B and C). To further correlate between ploidy levels and cell cycle phases, we supplemented the PI-generated profiles with those generated through Hoechst-Pyronin Y staining. The various cell cycle phases of euploid and aneuploid populations were gated based on their DNA content as indicated (Fig. 2B), and included—P1 (euploid G0), P2 (aneuploid G0), P3 (euploid G1), P4 (aneuploid G1 + euploid G2/M), and P5 (aneuploid S + G2/M). Combinatorial PI and Hoechst-Pyronin Y analysis clearly revealed the lack of an S phase within the aneuploid population along with the persistence of large fractions of cells in G1 and G2/M phases, thereby suggesting the subsistence of proliferation arrest within the aneuploid compartment of tumors (Fig. 2C). Most significantly, a distinct fraction of quiescent (G0) aneuploid cells was consistently detected in all the tumors analyzed.

To confirm the correlation of aneuploidy with cell cycle arrest, we examined the transcript levels of cell cycle inhibitors (p14, p16, p21, p27, and p57) and MDM2 (negative regulator of p53) within

Figure 2. Aneuploidy constitutes a nonproliferative subset within tumors. A, PI-based DNA content analysis of tumor-derived PKH subsets. The percentage distribution of different cell cycle phases within euploid and aneuploid fractions (bottom). B, representative contour plot for Hoechst-Pyronin Y staining–based cell cycle analyses; P1 to P5 demarcated distinct gates as indicated above the plot: P1, euploid G0 fraction; P2, aneuploid G0; P3, euploid G1; P4, euploid S + G2/M and aneuploid G1; P5, aneuploid S + G2/M phases; columns, percentages of each fraction. C, columns, mean cell cycle phases within euploid and aneuploid fractions identified through combinatorial analyses of PI and Hoechst-Pyronin Y staining; bars, contributions of the three PKH subsets to these phases. D, real-time PCR to detect expression levels (ΔCt expression values) of cell cycle checkpoints in the three PKH subsets; β-actin used as an internal control (*, P < 0.05).
the three PKH67-demarcated subsets, p16—G1-S and p14—G2/M arrest, respectively (28, 29), were found to be elevated in the PKHlo subset; significant upregulation of p27 in the PKHhi CSCs and not by the PKHneg cells provided the subtle distinction between cell cycle exit of quiescent PKHhi versus that of differentiated PKHneg subsets (Fig. 2D). Final validation of the proliferation inability of the tumor-derived aneuploid cells within the PKHlo subset came from their lack of proliferation (Supplementary Fig. S2D) and low clonogenic index when brought into culture (Supplementary Fig. S2E). The former was derived from the fact that upon sorting the PKHlo subset from A4 tumors (n = 3), and maintenance in culture, a complete depletion of the aneuploid fraction was evident within a few passages. Collectively, these results identify a proliferation arrest in the aneuploid cell fraction in tumors. This data is in concordance with recent studies that identify a negative role for aneuploidy in cellular proliferation (13).

Chemotherapy leads to the enrichment of quiescent CSCs that retain their functionality upon removal of selective pressure. The identification of CSCs as LRCs that undergo reversible quiescence provided the first indication of their involvement in tumor dormancy. Furthermore, the survival and persistence of their functionality during therapeutic regimes would provide a defining validation. We thereby assessed the outcome of chemotherapy on the quiescent, label-retaining PKHhi subset in drug-treated tumors. NOD/SCID mice injected (s.c) with PKH-labeled A4 cells were subsequently administered paclitaxel under two treatment regimes,
i.e., short-term and long-term exposures (STDE and LTDE, respectively; Fig. 3A, left), whereas vehicle control groups were treated with DMSO (Materials and Methods). Drug effects were not evident in STDE, because the tumor size remained comparable to those in the controls (Fig. 3A, right). However, under LTDE, tumors stopped growing, and marginally reduced in size as compared with those at 3 weeks indicating long-term cytotoxic effects. Thus, higher treatment efficacies are suggested in the LTDE over STDE group.

If the quiescence of CSCs operates like a safeguard mechanism against therapy, drug exposure could lead to the accumulation of LRCs. Our results conform to this reasoning as paclitaxel administration augmented PKHhi cells within STDE and LTDE tumors (Fig. 3B, left and right). It is likely that the elevated numbers of PKHhi CSCs in STDE tumors represent an initiation of drug-induced enrichment that is further enhanced on continuing treatment in LTDE tumors. We had earlier determined that the PKHhi label is retained for up to three to four divisions in vitro, after which it undergoes quenching to enter the PKHlo fraction (Supplementary Fig. S3A). An active enrichment of CSCs involving self-renewal through a few minimal divisions is thus possible without inducing sufficient PKH label quenching that could drive these cells into the PKHlo subset. Cell cycle analyses (PI staining) of A4-derived STDE and LTDE tumors showed enhanced G0-G1 in the euploid fraction (Fig. 4A, left); consequent Hoechst-Pyronin Y analyses elucidated that a significant amount of increase localized to the G0 rather than in the G1 phase (Fig. 4A, right). This analysis further supported the fact that tumor treatment leads to the accumulation of euploid cells in a quiescent state. Subsequent clonogenicity analysis of sorted PKHhi cells from STDE and LTDE tumors showed posttherapy reversion of drug-induced quiescence in tumor-derived PKHhi cells that retained their functionality (Supplementary Fig. S4A). This, along with a significant increase in the PKHhi subset in drug-exposed tumors supports our perception that the augmentation of quiescent cells within drug-treated tumors involves active enrichment of functional CSCs and is not a consequence of drug-mediated accumulation of label-retaining

Figure 4. Chemotherapy triggered proliferation within the aneuploid compartment. A, left, representative histograms of PI-based DNA content analysis of treated and control tumors; right, representative Hoechst-Pyronin Y staining dot plot of A4 untreated or treated tumors. B, columns, cell cycle phase frequencies within aneuploid fractions identified through combinatorial PI and Hoechst-Pyronin Y staining. C, columns, clonogenic potential of aneuploid fractions P2 and P5 from untreated and paclitaxel-treated tumors (*, \( P < 0.05 \)). D, representative DNA content analysis of the sorted and cultured P1 (euploid G0), P3 (euploid G1), P2 (aneuploid G0), and P5 (aneuploid S + G2/M) fractions from paclitaxel-treated tumors.
senescent or apoptotic cells. Recently, the role of transcription factors Snail and Slug in enriching CSCs under radioresistance and chemoresistance was reported from our lab (28); chemoresistant A4 cells (termed A4CR, resistant to 400 ng of paclitaxel; LD₃₀ for parental A4 cells was 60 ng) were shown to harbor higher frequencies of CSCs as compared with parental cells at steady-state. In the current study, we identified that these drug-resistant cells also exhibited a higher label-retention capacity in vitro (Supplementary Fig. S4B) and in vivo (Fig. 3C); however, A4CR-derived tumors under a paclitaxel regime do not undergo further LRC enrichment and were not growth-restricted (size) as compared with control tumors (Fig. 3D). This reflects a drug-tolerant stage under which chemoresistant cells achieve steady-state tumor growth.

Chemotherapy coaxes aneuploid cells out of their quiescent/proliferation-arrested states due to selective pressure. Our observations in developing tumors were that aneuploid cells tend to accumulate in the PKHlo subset either due to quiescence or proliferation arrest. This identifies them as potential cellular candidates that could contribute to tumor dormancy, survival, and regeneration. In assessing this postulate, we studied the outcome of chemotherapy on these cells in drug-treated tumors as above (STDE and LTDE). Although the number of aneuploid cells seemed to decrease under both paclitaxel treatment regimes, cell cycle analyses revealed the emergence of a distinct S phase fraction within the aneuploid subset (Fig. 4A, left). Furthermore, Hoechst-Pyronin Y resolution identified a higher fraction of cells in G₀ and S - G₂/M phases (Fig. 4A, right). Combining these analyses, we concluded that a redistribution of aneuploid cells occurs upon exposure to paclitaxel, leading to an increase of cells in G₀ and S phases, and decreased G₁ and G₂/M phases (Fig. 4B). This is a major difference between control tumor-generated aneuploid cells that were likely to be arrested in G₀-G₁ or G₂/M phases (Fig. 2A), and those in paclitaxel-exposed tumors that seem to re-enter the cell cycle albeit at very low frequencies, whereas a major portion of these cells might be affected by the drug. The reduction of post-treatment aneuploidy in the PKHlo fraction may thus be attributed to a reinstating of proliferation-induced label quenching within this population that was previously trapped within the PKHlo subset (Supplementary Fig. S4C). A functional evaluation of this potential in aneuploid fractions P2 (G₀) and P5 (S + G₂/M) sorted from paclitaxel-treated tumors based on Hoechst-Pyronin Y staining, identified increased clonogenic potential as compared with untreated tumors (Fig. 4C). Further profiling the DNA content of...
colonies formed in vitro by these fractions confirmed the cycling of aneuploid clones (Fig. 4D).

Discussion

Quiescence prevents stem cell exhaustion in normal tissues (30). In contrast to other nonproliferative states (terminal differentiation/senescence), only quiescent stem cells retain the ability to resume proliferation (31). Thereby, quiescence and its reversibility become defining parameters of stem cells. These characteristics have been speculated on extensively in the context of CSCs, especially with regard to drug evasion, but has never actually been experimentally shown. Thereby, the objectives of the present study were to (a) develop a convenient readout for the identification of quiescent cell fractions in an experimental model of tumor development, (b) functionally characterize these quiescent populations that distinguish them from other nonproliferative states, and (c) analyze their effects under drug treatment.

Through a convenient readout of label retention, we thus showed that stem cell activity is enriched in the quiescent fraction of a tumor that shows the capability to revert to a state of self-renewal and regeneration. Classically, CSC isolation from tumors exploits the detection of cell surface markers associated with normal stem cells. However, disparities in such approaches have been noted (32, 33), especially because quiescence has not been shown in these cells. Moreover, although surface markers provide obvious advantages, a single phenotype cannot account for the heterogeneity within CSC populations (34–36). Our results further indicate that a specific surface expression may not be exclusively associated with CSCs, but also encompasses progenitor and aneuploid populations. In understanding the events that lead to tumor dormancy, it is imperative to subject putative CSCs to a functional assessment such as quiescence and/or drug resistance capabilities rather than surface phenotypes. Besides harboring CSCs, most solid tumors contain aneuploid populations that represent genetically diverse cell pools with reduced cell proliferation and cellular fitness (13). In a small subset of such cells, however, cellular imbalances associated with increased mutation rate, gene amplification, and/or genomic instability have linked aneuploidy with tumor dormancy (37, 38). On this background, our observations clearly illustrate (a) the stem-like potential of tumor-derived LRCs to survive, persist, and retain functionality through drug regimes; thereby contributing to tumor dormancy; and (b) acquisition of proliferation potential by quiescent/cell cycle–arrested aneuploid cells through a re-entry into the cell cycle to contribute to disease progression. Chemotherapy-imposed selective pressures could trigger this response which exploits intrinsic survival capabilities to finally result in a phenotypic switch. Additional acquisition of stem-like characteristics by such a clone ensures its long-term survival and potential contribution to tumor dormancy (Fig. 5).

Prompted by the detection of LR-CSCs within tumors, we also attempted to detect LRCs in cancer cell lines in vitro. Towards this end, the A4 cells were membrane-labeled with PKH, cultured and harvested at regular intervals (48 hours) for analyses of label intensities by flow cytometry. By 78 hours, a large bulk of cells underwent quenching of label intensity (Supplementary Fig. S3A). Two groups could be demarcated within the bulk cells based on differential label intensities that correspond with different proliferation rates; we termed these two populations as lagging (slow) and leading (fast) groups (Supplementary Fig. S4A and B). However, these subtle proliferative differences in vitro did not seem to have the same significance as quiescent cells in situ, as further monitoring revealed that the entire population of cells in culture underwent a complete quenching of label by 264 hours (Supplementary Fig. S4A). In addition to A4 cells, we also analyzed the proliferation profiles of NT2, C6, U87, HL60, PA1, and T47D cell lines. All these cells, under defined culture conditions, showed an analogous pattern of proliferation marked by the absence of LRCs/PKHlow subset (Supplementary Fig. S3C). Thus, in contrast to their growth in situ, cultured cells seem to be devoid of LRCs, probably due to use of culture conditions optimized for cell proliferation. As a corollary, tumor-derived quiescent PKHth cells, when brought into culture, showed a high clonogenic capability. The intrinsic quiescence of cells in vitro is however not irreversibly lost, but can be recapitulated in situ where niche elements exert their instructive role. Thereby, the “stem-like” cells in tumors result from effects of intrinsic and extrinsic elements during tumor formation. A similar situation is observed in case of aneuploid cells that are associated with developing tumors and do not proliferate rapidly in culture. Our observations receive support from previous studies proposing that the reciprocal interaction between the tumor cells and microenvironment controls the switch between proliferation and quiescence (3, 39, 40).

Quiescent LR-CSCs undergo intermittent divisions leading to self-renewal and generation of rapidly proliferating TA progenitors (that reside within the PKHth subset and subsequently give rise to the bulk tumor—PKHlow cells; Fig. 5). Genetic instability during such divisions may also generate aneuploid CSC progenitor clones (38). These aneuploid populations, akin to the CSCs, also constitute a dormant subset within tumors either through quiescence or a proliferation arrest. Therapy may eradicate TA cells because most drugs target proliferative cells; concurrently, the differentiated bulk tumor cells also perish, ultimately leading to tumor shrinkage. Thus, drug exposure leads to two possibilities—a dormant aneuploid clone refractile to drug effects might emerge to drive aggressive disease progression, or remission ensues, reflecting an initial high therapeutic efficacy. However dormant, therapy-refractory CSCs and aneuploid CSCs may persist within the host as minimal residual disease. The triggering of these cellular elements defining tumor dormancy from their state of quiescence is not understood as yet, but their capability to lead to disease relapse is recognizable from the results presented here.

Our findings also indicate that the cellular dormancy program in tumor-derived LR-CSCs and aneuploid cells might be dominant over oncogenic signaling, thereby ensuring their survival in a quiescent state. Tumor dormancy complements the selection of a chemoresistant clone that is likely to be aneuploid to provide the genetic variability for drug-resistant growth and acquisition of stem cell characteristics. Several mechanisms are possibly involved, e.g., expression of multidrug resistance proteins, increased resistance to drug-mediated apoptosis, tumor stroma–mediated acquisition of drug resistance, etc. (41–43). From a clinical perspective, our findings illustrating the contribution of CSCs and aneuploid populations to tumor dormancy has significant therapeutic implications, but presents a major challenge because the multidrug resistance exhibited by the CSCs and unstable karyotype confers intangible, adaptive advantages to aneuploid cells. Thus, it becomes increasingly important to further decipher the interactions and systemic signals that govern the maintenance of the dormant state and the switch between dormancy and proliferation in these cells, including exploiting the putative niche that nurtures the CSCs and their aneuploid progeny as drug targets.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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