Asymmetric cancer cell division regulated by AKT

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Human tumors often contain slowly proliferating cancer cells that resist treatment, but we do not know precisely how these cells arise. We show that rapidly proliferating cancer cells can divide asymmetrically to produce slowly proliferating “G0-like” progeny that are enriched following chemotherapy in breast cancer patients. Asymmetric cancer cell division results from asymmetric suppression of AKT/PKB kinase signaling in one daughter cell during telophase of mitosis. Moreover, inhibition of AKT signaling with small-molecule drugs can induce asymmetric cancer cell division and the production of slow proliferators. Cancer cells therefore appear to continuously flux between symmetric and asymmetric division depending on the precise state of their AKT signaling network. This model may have significant implications for understanding how tumors grow, evade treatment, and recur.

Tumors generally evolve through years of mutation and clonal selection (1). This favors the outgrowth of rapidly proliferating cancer cells over time. However, even advanced tumors contain many cancer cells that appear to be proliferating slowly (2). This proliferative heterogeneity correlates closely with time to clinical detection, growth, metastasis, and treatment response across all tumor types, but we still do not understand clearly how it arises. The rate of mammalian cell proliferation is generally determined by the time spent in G1 of the cell cycle. Critical genetic and epigenetic changes within cancer cells accelerate G1 transit, whereas a suboptimal microenvironment with imbalance of growth factors, nutrients, or oxygen can slow G1 progression (3). Therefore, individual cancer cells within a tumor are thought to vary significantly in their proliferative rate depending on the precise balance of these intrinsic and extrinsic factors. Interestingly, however, many tumor-derived cancer cell lines also produce slowly proliferating cells. These established lines have many acquired mutations that drive cell proliferation. They have also been grown ex vivo for years in a stable microenvironment to promote unbridled proliferation. These factors often favor a strong purifying selection against slow proliferators. We worked to understand how slowly proliferating cells seem to arise paradoxically in cancer cell lines.

Results

G0-Like Cancer Cells in Vitro. We began by studying MCF7, a highly proliferative, aneuploid, ER7/HER2 human breast cancer cell line. This line displays significant proliferative heterogeneity despite mutations in CDKN2A and PIKK3CA that cooperatively drive cell-cycle progression (4). We first hypothesized that slowly proliferating MCF7 cells might produce low levels of reactive oxygen species (ROS). This hypothesis was based on previous observations that slowly cycling hematopoietic, neural, and breast adult stem cells and cancer stem cells produce low levels of ROS (5–7). We stained MCF7 cells with 5-(and-6)chloromethyl 1,2,7-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA), a live-cell dye for ROS, and used fluorescence-activated cell sorting (FACS) to isolate ROSlow and ROShigh cells (which we defined as the bottom and top 1% of the observed distribution, respectively).

Indeed, cell-cycle analysis confirmed that ROSlow cancer cells were predominantly in G1/G0 of the cell cycle compared with actively cycling ROShigh cells (Fig. L4). ROSlow cells did not alter expression of ESR1 or MYC, which are both necessary for MCF7 proliferation (Fig. 1B and Fig. S1A) (4, 8). However, these cells expressed very low levels of critical proliferation proteins (e.g., MKI67low, MCM2low, CDC6low, GMNNlow, AURKA low, PLK1low) and histone marks (i.e., H3S10phlow, H3K4me2low, H3K9me2low, H3K27me3low, H4K12ac low, H4K16ac low) that are suppressed in noncycling cells (Fig. 1 C–E and Fig. S1A) (2, 9–12). Notably, ROSlow cells were not unique to MCF7; we could also find similar slowly cycling cells in the MDA-MB-231 breast and HCT116 colon cancer lines (Fig. S1B).

Molecular Profiling of G0-Like Cells. We next used two different molecular profiling approaches to further characterize ROSlow cancer cells. Microarray transcriptional profiling revealed that ROSlow MCF7 and HCT116 cells modestly up-regulated the HES1 transcription factor (false discovery rate (FDR) = 0.05, fold change = 2.8) (Fig. 1 F and G). This was unexpected because HES1 was recently reported to uniquely mark normal quiescent fibroblasts that have completely withdrawn from the cell cycle into a G0 state (13). We therefore considered the provocative possibility that ROSlow cancer cells might actually be an “out-of-cycle” subpopulation in G0 (rather than prolonged G1). Within 24–48 h of culture in vitro, however, we found that many ROSlow cells up-regulated proliferation proteins and histone marks, divided, and formed colonies normally (Fig. 1 H and J). This argued that ROSlow/HES1high cancer cells might express a special G0 marker but that they were, in fact, not quiescent. In addition, ROSlow cells did not express a cancer stem cell transcriptional or marker profile (e.g., CD44high/CD24low or VIMhigh/CD117low) (Fig. S1 C and D) (14, 15). We therefore chose the term “G0-like” to describe these slowly cycling ROSlow cancer cells.

We also used a reverse-phase peptide microarray approach to probe ROSlow G0-like cancer cells for alterations in common signaling pathways (16). We made microarrays with whole-cell lysates of ROSlow and ROShigh cells from MCF7 and HCT116. We then interrogated these arrays with 89 different, highly validated antibodies targeting a spectrum of cancer-relevant phosphoproteins and proteins (Table S1) (17). Statistical analysis of the


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Fig. 1. Slowly cycling G0-like cancer cells in vitro. (A) FACS analysis of MCF7 cells with gates for sorting ROS\textsuperscript{low} (blue) and ROS\textsuperscript{high} (red) cells. Insets show cell-cycle profiles of ROS\textsuperscript{low} and ROS\textsuperscript{high} populations. Tables indicate percentage of cells with respective DNA contents. (B–F) Cytospin of cells sorted for high or low ROS staining and stained for (B) ESR1, (C) MKI67, (D) MCM2, (E) H3K9me2, and (F) HES1. Merged images represent respective stains merged with underlying DAPI stain. (G) Heatmap of transcriptional profiles of three independent replicates of ROS-sorted MCF7 and HCT116 cells (columns). Rows depict expression of genes with greater than twofold change in expression and FDR < 0.25. Numbers on the right indicate fold change. Colorgram depicts high (red) and low (blue) relative levels of gene expression. (H) ROS-sorted cells stained for MKI67 and H3K9me2 either as cytospins (0 h) or after 24 h of culture. Merged images represent respective stains merged with underlying DAPI stain. (I) Plot shows number of colonies from MCF7, ROS\textsuperscript{low}, or ROS\textsuperscript{high} from three independent experiments (each symbol represents one independent experiment). Error bars indicate SD. me2, dimethyl.
Fig. 2. Molecular profiling of G0-like cells. (A) Heatmap of proteomic profiles of two independent replicates of ROS-sorted MCF7 and HCT116 cells (columns). Rows represent proteins with significant change in expression (FDR < 0.25). Numbers on the right indicate FDR. Colorgram depicts high (yellow) and low (blue) relative levels of proteins. (B and C) Cytospins of ROS-sorted cells stained for (B) pAKT (Ser473), pAKT (Thr308), pS6RP (235/236), and pS6RP (240/244) and (C) pan-AKT. (D) Western blot for pan-AKT on bulk and ROS-sorted MCF7 cells. p, phosphorylated.

resulting dataset identified 9 up- and 14 down-regulated protein markers in ROSlow compared with ROShigh cells (FDR < 0.25) (Fig. 2A). Interestingly, these differentially expressed proteins included important members of the AKT/PKB signaling pathway that suggested a down-regulation of AKT signaling in slowly cycling ROSlow cells (i.e., pS6RP, p70S6K, pFOXO1, pGSK3β, PRAS40) (18). Consistent with this inference, we found that ROSlow cells had low levels of both phoso-AKT (pAKT-T308 and pAKT-S473) and its downstream target phospho-S6RP (pS6RP-S235/236 and pS6RP-S240/244) (Fig. 2B and Fig. S1E). Furthermore, ROSlow cells also expressed low levels of total AKT protein itself (Fig. 2 C and D).

G0-like Cancer Cells Arise Through Asymmetric Division. AKT signaling plays a major role in promoting cell-cycle progression (18). We therefore wondered whether slowly cycling AKTlow G0-like cancer cells arise by suppressing AKT protein levels. To test this hypothesis, we overexpressed a cDNA for an AKT1-mCherry fusion protein in MCF7 cells to ask whether this would inhibit the formation of G0-like cancer cells. However, forced AKT overexpression did not appreciably change the frequency of these cells. Instead, G0-like cancer cells with low endogenous AKT levels also suppressed expression of exogenous AKT1-mCherry protein in both MCF7 and HCT116 cells (Fig. S2 A and B).

In addition, we noted that actively dividing MCF7 cells occasionally showed striking asymmetric expression of both the exogenous AKT1-mCherry and endogenous AKT protein, in contrast to most dividing cells, which appeared symmetric (Fig. 3 A–C). This asymmetric expression occurred exclusively in telophase (after formation of the nuclear membrane but before cytokinesis) in the thousands of dividing cancer cells that we examined. In asymmetric mitosis, one daughter was in a proliferative stance (e.g., H3K9me2high/HES1low) with diffuse AKT expression in both the nucleus and cytoplasm (Fig. 3B and D). Its sibling was in a G0-like posture (e.g., H3K9me2low/HES1high) with perinuclear HES1 expression and more intense nuclear localization of AKT (Fig. 3B and D). Importantly, G0-like cells in telophase that were AKT-nuclearhigh appeared to suppress AKT protein expression to become AKTlow after cell division (Fig. 3E). G0-like daughters in interphase also had higher levels of nuclear-localized FOXO1, a direct target of AKT signaling that localizes to the nucleus with loss of AKT signaling and can strongly suppress cell-cycle progression (Fig. 3F) (18). We did not observe any consistent differences in the size or general appearance of G0-like daughters. G0-like cancer cells therefore appeared to arise through occasional, asymmetric loss of AKT signaling (with nuclear localization and then suppression of AKT protein), resulting in the birth of a slowly cycling cancer cell.

Fig. 3. G0-like cancer cells arise through asymmetric division. (A–D) MCF7 cells in telophase stained for DAPI, β-tubulin (TUBB), (A) AKT1-mCherry cells, asymmetric, (B) pan-AKT, asymmetric, (C) pan-AKT, symmetric, and (D) HES1, asymmetric. (E and F) MCF7 cells in interphase stained for (E) DAPI, β-tubulin, H3K9me2, and pan-AKT and (F) DAPI, HES1, H3K9me2, and FOXO1.
Induction of Asymmetric Cancer Cell Division with AKT Inhibition.

Both MCF7 and HCT116 have activating mutations in PIK3CA that drive constitutive AKT signaling on which these cells depend for their survival (19). It was thus curious that G0-like cells found in MCF7 and HCT116 could tolerate suppression of AKT signaling. We therefore asked whether experimental suppression of AKT signaling could actually induce G0-like cancer cells rather than killing them. We treated MCF7 cells with a small-molecule, allosteric AKT-1/2 inhibitor (AKT-1/2i) for 72 h and looked for G0-like cells (20). As expected, low-dose AKT-1/2i (0.1 μM) had no appreciable effect on these cells, whereas a high dose (5 μM) induced significant apoptosis (Fig. 4A). Surprisingly, however, an intermediate dose of AKT-1/2i (2 μM) (which only partially inhibited AKT signaling; Fig. 4B) increased the number of MCM2high/H3K9me2high/HES1high G0-like cells from an estimated 1% at baseline to about 50% of the population (Fig. 4C and D). Furthermore, AKT-1/2i treatment also increased the frequency of asymmetric mitotic cells by approximately threefold (Fig. 4E). These increases were associated with a profound slowing of proliferation, which was completely reversible with inhibitor washout, but no appreciable cell death (Fig. 4F). We also obtained similar results in HCT116 cells and with a second, clinical-grade allosteric AKT-1/2 inhibitor (MK-2206) (Fig. S3 A–Q) (21). In contrast, we could not induce a similar G0-like marker profile in cells treated with 20 μM rapamycin (a specific inhibitor of the mTOR kinase, which is a direct downstream target of AKT), 10 nM staurosporine (a general protein kinase inhibitor), or 50 μM N-[(3,5-difluorostyryl)acetyl]-1-amyl-2-phenyl]glycine-1,1-dimethylethyl ester (DAPT) (an inhibitor of the γ-secretase/NOTCH pathway that regulates HES1), which each inhibited MCF7 cell proliferation by ~50% but did not induce G0-like cells.

We also performed live-cell imaging experiments to examine the dynamics of cancer cell division with and without AKT-1/2i treatment. We first obtained serial images of MCF7 cells dividing over time. We then analyzed these images to identify individual cells, created lineage traces of these individual cells and their progeny to identify sibling pairs, and measured intermitotic times for each cell. We found that AKT inhibition slowed the average cell-cycle time of MCF7 cells from 26 to 49 h and dramatically increased the fraction of slowly cycling cells. For example, 1% of cells had a cell-cycle time of >100 h at baseline, whereas AKT-1/2i treatment increased this fraction to about 10% (Fig. 4G). Furthermore, this increased proliferative heterogeneity was associated with an increase in asymmetric division. Sibling–sibling differences in time to next mitosis increased from about 6.2 to 16.9 h on average. Whereas 2% of sibling cells had a difference in time to next mitosis of >60 h at baseline, AKT-1/2i treatment increased this fraction to about 10% (Fig. 4H and Movie S1). In addition, AKT-1/2i induced asymmetric division more potently when first delivered to cells just before mitosis rather than at other points in the cell cycle (Fig. 4I). These results suggested that quantitative inhibition of AKT signaling induced asymmetric division with respect to the proliferative potential of daughter
cells, and that the precise timing of AKT signaling loss in mitosis was important for this effect.

**G0-Like Cells Are Enriched After Cytotoxic Treatment in Vivo.** Our findings demonstrated that rare, slowly cycling G0-like cells were present in established human cancer cell lines. Moreover, these G0-like cells appeared to down-regulate AKT protein and signaling. We therefore asked whether we could find similar AKT\textsuperscript{low}\ G0-like cancer cells in actual human tumors in vivo. We focused on patients with newly diagnosed breast tumors who were given neoadjuvant chemotherapy before definitive surgical resection of their tumor. This scenario enabled us to examine matched tumor biopsies that were obtained from individual patients before and after treatment with multiple cycles of adriamycin, cyclophosphamide, and paclitaxel chemotherapy (Table S2).

Interestingly, we found occasional MCM2\textsuperscript{low}/H3K9me2\textsuperscript{low}/HES1\textsuperscript{high} G0-like cancer cells that expressed low levels of total AKT protein in the pretreatment biopsies from each of the five patients we examined (Fig. 5A and C–E and Fig. S4 A and B). These cells were not localized in any appreciable histopathologic pattern but were present across all three molecularly distinct subtypes of breast cancer (ER\textsuperscript{+}, ERBB2\textsuperscript{+}, ER/ERBB2\textsuperscript{+}). Furthermore, we found a striking enrichment in these AKT\textsuperscript{low}/MCM2\textsuperscript{low}/H3K9me2\textsuperscript{low}/HES1\textsuperscript{high} cells in matched biopsies obtained after treatment from all five patients (Fig. 5B and C–E and Fig. S4 A and B). This clinical experiment suggested that slowly cycling G0-like cancer cells could indeed be visualized using the AKT\textsuperscript{low}/MCM2\textsuperscript{low}/H3K9me2\textsuperscript{low}/HES1\textsuperscript{high} molecular profile in patients with breast cancer, where they appeared to survive intensive exposure to combination chemotherapy. Additional experiments in vitro further suggested that G0-like cancer cells indeed appeared to survive exposure to cytotoxic insult (Fig. S5).

**Discussion**

Our results suggest that rapidly proliferating cancer cells occasionally suppress AKT signaling asymmetrically during mitosis to produce a slowly cycling G0-like daughter cell with a ROS\textsuperscript{low}/MKI67\textsuperscript{low}/MCM2\textsuperscript{low}/H3K9me2\textsuperscript{low}/HES1\textsuperscript{high}/AKT\textsuperscript{low} profile (Fig. S6). Asymmetric inhibition of AKT signaling in one emerging daughter cell is associated with nuclear localization followed by suppression of AKT protein and proliferative arrest of this newborn cell. Furthermore, inhibition of AKT signaling modulates the MKI67, MCM2, H3K9me2, and HES1 marker profile while also having a well-described role in regulating cellular ROS levels that both coordinately mark G0-like cancer cells (22–24). G0-like cancer cells are not stably quiescent in cell culture, but rather tend to re-enter the cell cycle within days. Nevertheless, the G0-like cells that we find in breast cancer patients appear to be highly enriched after 6 mo of exposure to combination chemotherapy. This suggests that G0-like cells might be able to maintain a stable “out of cycle” state for a longer period of time in vivo. We do not yet know whether G0-like cells in vivo are indeed regulated by AKT suppression as in vitro. Nor do we know whether G0-like cancer cells are enriched in vivo primarily through selection or induction by chemotherapy. However, many factors in the complex tumor microenvironment, including exposure to chemotherapy, modulate AKT signaling, leading us to speculate that AKT modulation (either naturally occurring or pharmacologically induced) may in fact regulate the proportion of G0-like cancer cells within actual human tumors (25, 26).

Interestingly, two recent reports have identified rare, slowly cycling subpopulations in lung and melanoma cancer cell lines that are drug resistant, but it is unclear whether these cells similarly arise through asymmetric division (27, 28).

The asymmetric cancer cell division that we observe is not a special property of a discrete subpopulation. Rather, it appears to be a latent but general property of any cancer cell that can be dynamically unmasked depending on the precise state of its AKT signaling network. Presumably, both intrinsic and extrinsic factors that modulate AKT signaling can shift the dynamic between symmetric and asymmetric division. These observations may therefore open previously unappreciated experimental opportunities for studying asymmetric division in mammalian cell culture, a long sought after but unrealized goal (29). It is critical to note that this asymmetric cancer cell division which we describe does not relate to the generation of cells with a different size or fate, as

![Fig. 5. G0-like cells enriched after treatment in vivo. Human breast tumor samples from five different patients were stained for H3K9me2, MCM2, HES1, pan-AKT, and cytokeratin. G0-like cytokeratin-positive cells (defined as H3K9me2\textsuperscript{low}/MCM2\textsuperscript{low}/H3K9me2\textsuperscript{low}/HES1\textsuperscript{high}/AKT\textsuperscript{low}) before and after chemotherapy were counted. (A and B) Human breast tumor from patient 1 stained for DAPI (blue), dimethyl-H3K9 (green), human cytokeratin (yellow), and pan-AKT (red) (A) before (pretreatment) and (B) after (posttreatment) chemotherapy. The arrow points to G0-like cells. (C–E) Bar graph of percentages of H3K9me2\textsuperscript{low}/MCM2\textsuperscript{low}/H3K9me2\textsuperscript{low}/HES1\textsuperscript{high}, and H3K9me2\textsuperscript{low}/pan-AKT\textsuperscript{low} cytokeratin-positive cells before (red) and after (blue) chemotherapy for the five patients.](image-url)
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Removing the American Type Culture Collection (ATCC). Cell culture, clonogenic assays, and drug treatment were performed using established protocols. All experiments were performed using standard cell culture conditions. Fluorescence activated cell sorting (FACS) (on BD FACSCalibur, LSRII, FACSARia, and FACSARia II cell sorters), immunofluorescence imaging (on a Nikon Eclipse Ti A1R-A1 confocal microscope), and live cell imaging (on the Nikon Biostation CT platform) were performed using established methods and standard protocols. Transcriptional profiling was performed using the Affymetrix IVT express kit and Human Genome U133 Plus 2.0 microarrays (Affymetrix). Reverse-phase protein microarray profiling and Western blotting were performed using antibodies listed in Table 1. Full details can be found in SI Materials and Methods.

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Materials and Methods

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