Alternative to the soft-agar assay that permits high-throughput drug and genetic screens for cellular transformation

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Colony formation in soft agar is the gold-standard assay for cellular transformation in vitro, but it is unsuited for high-throughput screening. Here, we describe an assay for cellular transformation that involves growth in low attachment (GILA) conditions and is strongly correlated with the soft-agar assay. Using GILA, we describe high-throughput screens for drugs and genes that selectively inhibit or increase transformation, but not proliferation. Such molecules are unlikely to be found through conventional drug screening, and they include kinase inhibitors and drugs for noncancer diseases. In addition to known oncogenes, the genetic screen identifies genes that contribute to cellular transformation. Lastly, we demonstrate the ability of Food and Drug Administration-approved noncancer drugs to selectively kill ovarian cancer cells derived from patients with chemotherapy-resistant disease, suggesting this approach may provide useful information for personalized cancer treatment.

cancer | transformation | drug screen | genetic screen | personalized medicine

For nearly 50 y (1, 2), the gold-standard assay for cellular transformation/tumorigenicity has been the soft-agar assay. This classic assay requires that cells grow in an anchorage-independent manner, a hallmark of cancer cells, but not normal cells; it was a significant improvement over earlier assays that involved growth in suspension on "bacterial" plates. Importantly, growth in soft agar is strongly correlated to tumorigenicity in animals, typically mouse xenografts. Compared with cells grown in 2D monolayers attached to plates, such 3D growth conditions more accurately reflect the natural environment of cancer cells (3) and are crucial to be performed before animal studies (4, 5). However, the soft-agar assay is slow, labor-intensive, imprecise, inconsistent because of subjective definitions of colonies, and not suitable for high-throughput screens. Unlike nontransformed cells, transformed cells can grow in multiwell plates that are coated with poly(2-hydroxyethyl methacrylate) (poly-HEMA) that prevent cell attachment to the surface (6). However, it is unclear whether growth on such low-attachment plates is comparable to the soft-agar assay, because few cell lines were tested and quantitative analysis was not described.

Although it is well recognized that different culture plate surfaces promote distinct growth characteristics of malignant and nontransformed cells, drug and genetic screens are routinely performed on standard plates that permit efficient attachment. While this work was in progress, a proof-of-principle screen of 89 known anticancer compounds involving the comparison of growth on low- and high-attachment plates was described (7). However, this study did not screen unknown drugs, nor did it describe the use of low-attachment plates for genetic screens. In addition, this study used nontransformed and transformed cell lines, not cells from cancer patients.

Every person's cancer is different with respect to the constellation of mutations, methylated tumor suppressor genes, and epigenetic states. As a consequence, the phenotype of every individual cancer is unique, particularly the response of a patient to drugs. As such, we and others have been interested in developing methods for personalized medicine, in which tumor cells from individual patients are tested for sensitivity to a panel of drugs. Furthermore, many drugs that are in clinical use for other diseases (e.g., metformin for diabetes, various antiinflammatory drugs, simvastatin for heart disease) have anticancer effects in vitro (8) and, hence, have the potential to be repurposed for treating cancer patients. For these reasons, we wish to screen patient-derived tumor cells for their response to a variety of Food and Drug Administration (FDA)-approved drugs by using an assay that is specifically relevant for cancer cells.

Here, we describe a modified assay in which growth in low attachment (termed GILA) is quantitated by measuring ATP in permeabilized cells. We demonstrate that GILA is a rapid and quantitative assay for cellular transformation that correlates strongly with the soft-agar assay. We perform high-throughput drug and genetic screens for drugs or genes that, respectively, inhibit or increase transformation. Lastly, we identify drugs that selectively inhibit the growth of fresh, patient-derived ovarian cancer cells that are refractory to conventional chemotherapeutic treatment, indicating the potential of this approach for personalized medicine.

Results

The GILA Assay Is Comparable to the Soft-Agar Assay for Cellular Transformation. The principle of GILA is that transformed cells can grow on low-attachment plates, whereas nontransformed cells cannot. A critical parameter for the GILA assay is cell density

Significance

The paper describes an assay for cellular transformation that involves growth in low attachment (GILA). This assay is comparable to the gold-standard soft-agar assay, but it is much easier to perform and is suitable for high-throughput drug and genetic screens. We describe such screens for drugs and genes that selectively inhibit or increase transformation, but not proliferation. Such molecules are unlikely to be found through conventional drug screening. Lastly, we demonstrate the ability of Food and Drug Administration-approved noncancer drugs to selectively kill ovarian cancer cells derived from patients with chemotherapy-resistant disease, suggesting this approach may provide useful information for personalized cancer treatment.

Author contributions: A.R. and K.S. conceived of the strategy; A.R., A.J., and K.S. designed the experiments; A.R. and A.J. performed GILA and high-attachment assays; J.G.D. designed the genetic screen; A.R. performed drug and genetic screens; Z.J. analyzed the genetic screen and designed the validation experiment; A.R. and B.I. performed and K.S. and L.A.G. coordinated the ex vivo drug sensitivity experiments; K.S. was responsible for the overall project management and supervision; and A.R. and K.S. wrote the paper.

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(number of cells per well), and this density needs to be determined empirically. For the cell lines used here, this density was optimized at 1,000 cells per well in 100 μL of medium for 96-well plates or 50 cells per well in 30 μL of growth medium for 384-well plates. We examined 13 cell lines originating from four different tissues—fibroblast, breast, ovary, and prostate (Fig. S1). An equal number of cells from each line were seeded into wells of an ultralow attachment 96-well plate, grown for 5 d, and assayed for ATP levels as a surrogate for number of viable cells. As expected, the transformed cells grew significantly better than nontransformed cells (Fig. S2), with 5- to 30-fold increased ATP levels (Fig. 1).

To examine whether GILA was comparable to the soft-agar assay, we performed soft-agar assays on the same cell lines. As expected, a large number of big colonies developed from transformed cells, whereas nontransformed cells generated few small size colonies and mostly individual resting cells (Fig. S2). When transformation values of both assays were plotted, the linear correlation coefficient value is 0.68 (Fig. 1), a strong correlation indicating that GILA and the standard soft-agar assay are essentially comparable assays for transformation.

The GILA Assay Specifically Measures Cellular Transformation. To confirm that GILA is a measure of transformation and not simply growth per se, we compared the growth of cells as a monolayer on a traditional high-attachment surface to 3D growth in the GILA assay (Fig. 2). Proliferation rates on high-attachment conditions correlate poorly with those on low-attachment conditions (R^2 = 0.12). In fact, with the exception of fibroblasts, nontransformed cells grow faster than transformed cells on high-attachment conditions, the opposite situation from low-attachment conditions (Fig. S3). These observations indicate that GILA specifically measures oncogenic capacity of transformed cell lines independently from the proliferation rate.

A GILA-Based Drug Screen in Transformed Fibroblasts Identifies Antineoplastic Compounds Overlooked by a Conventional Screen. Most high-throughput screens for small molecules or genes that have antitumorigenic or protumorigenic roles are performed under standard growth conditions on high-attachment surfaces. As such, the candidates passing these screens may inhibit or enhance general proliferation but not necessarily have a specific

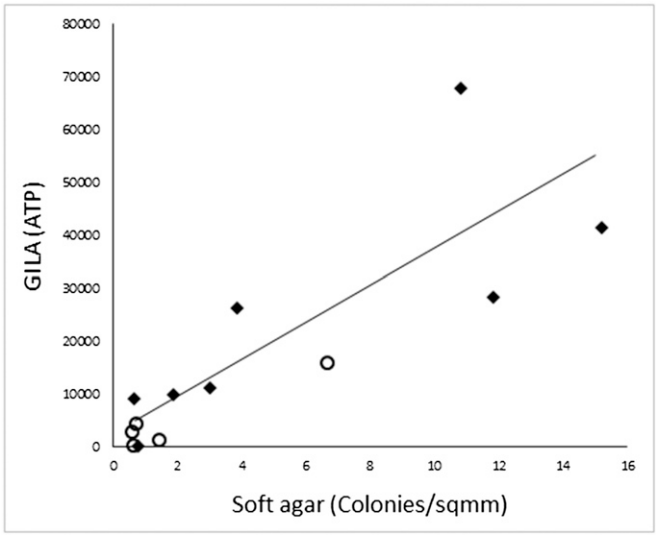

Fig. 1. Correlation between soft agar and growth in low-attachment assay. Scatter diagram of transformation values for transformed (black diamonds) and nontransformed (open circles) cells as obtained from both SA and GILA assays. Each point represents three independent repeats of each assay for cell line.

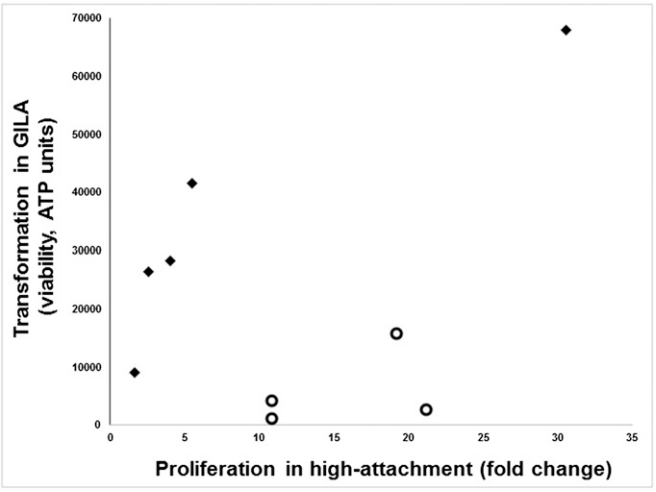

Fig. 2. Anchorage-dependent growth of cells is not correlated to anchorage-independent growth. Proliferation values of viable cells after 5 d of anchorage-dependent growth and the transformation values of these cells. Note that four of the top five rapid growing lines are nontransformed cell lines (open circles), whereas most of the transformed lines (black diamonds) show slower proliferation. Each experiment had three independent repeats.

effect on transformation or cancer cells. To identify drugs specifically inhibiting the oncogenic state of fibroblasts transformed with Harvey Rat Sarcoma viral oncogene homolog (H-RAS) (9), we used GILA as a primary screen and growth on standard high-attachment plates as a counterscreen. Drugs specifically affecting the transformed state would inhibit anchorage-independent growth as assayed by GILA, but have little effect on high-attachment conditions.

Among the 633 kinase inhibitors and FDA-approved drugs screened, we identified 10 compounds that significantly inhibit cell growth in the GILA assay, while having a less pronounced inhibitory effect on the high-attachment surface (Fig. 3*A* and Fig. S4). These 10 compounds were subject to a validation (secondary) screen. Four drugs inhibited cell growth exclusively in the GILA assay, five drugs preferentially inhibited growth in low-attachment conditions will small inhibitory effects on high-attachment growth (~20% reduction), and one drug inhibited growth to comparable extents in high- and low-attachment conditions (~50% reduction). These drugs would probably have been ignored in a conventional screen for growth, but their ability to inhibit growth on low-attachment surfaces (reduction of 20–80% at the concentrations tested) make them interesting candidates with specific antineoplastic activity (Fig. 3*B*). As expected, the five candidate drugs tested inhibit colony formation in the soft-agar assay (Fig. 3*C*).

Drugs used for treatment of diabetes, atherosclerosis, and inflammatory disease often inhibit transformation and tumor growth, and these diseases have similarities in their transcriptional signatures (8). In this regard, five of the top hits from our drug screen have never been shown to play a role in cancer and, hence, might be interesting candidates to repurpose for cancer therapy. In contrast, three drugs (calcipotriol, sibutramine, and nitazoxanide) have targets that affect pathways that overlap with cancer-related pathways (10). For example, the antiviral drug nitazoxanide suppresses IL-6 (11), a cytokine frequently linked to carcinogenesis. Lastly, a kinase inhibitor (STK855495) inactive in other cell-based assays to date inhibits cellular transformation and, hence, is of interest for further study.

Patient-Derived Ovarian Cancer Cells Are Sensitive to FDA-Approved Drugs Identified in the GILA-Based Screen. To test whether drugs identified and validated in the drug screen had antineoplastic

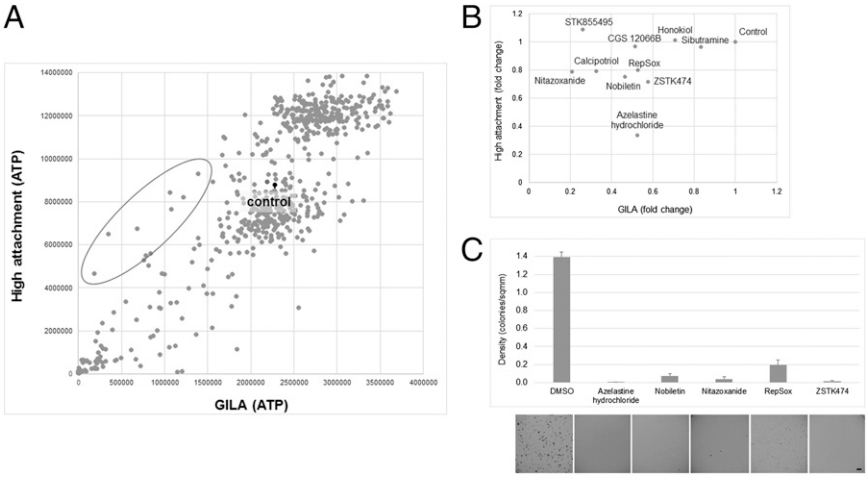

Fig. 3. GILA for screening small molecules for antineoplastic activity. (*A*) Kinase inhibitors and FDA-approved drugs were screened for their ability to affect transformed cells growing either on high- or low-attachment surface. Among the 633 drugs, 10 drugs (circled) were found to be selectively effective against cells that were growing on a low-attachment surface. Control cells are marked in black. (*B*) Secondary screen to the 10 hits confirmed the specific inhibitory role of the compounds in anchorage-independent growth (GILA). (*C*) Validation of candidate drugs in transformed cells by the soft-agar assay. (Scale bar: 250 μm.)

activity in the context of human disease, we isolated fresh, ascites-derived ovarian cancer cells from five patients who had failed multiple lines of treatment (Fig. S5). Immediately after procurement, ascites samples were processed and 5,000 cells per well were flow-sorted into 96-well microtiter plates with low- or high-attachment surfaces containing 100 μL of growth medium per well. These ex vivo cultures were treated individually with four drugs identified and validated in the drug screen (azelastine hydrochloride and nitazoxanide, RepSox, or nobiletin) for 72 h. Two compounds, azelastine hydrochloride and nitazoxanide, demonstrated inhibitory activity in the GILA assay (Fig. 4*A*), with a dose-dependent response shown for azelastine hydrochloride (Fig. 4*B*). Interestingly, cancer cells from the various patients displayed differential sensitivity to these drugs. These results validate ex vivo GILA-based drug screens as a potential tool for drug discovery and repurposing to individually treat cancer patients who have failed to respond to conventional treatment.

A Genome-Scale ORF Screen Identifies Genes That Specifically Increase Transformation. We used GILA in a genetic screen for oncogenes by introducing a barcoded library of lentiviruses overexpressing protein-coding regions (18,000 clones representing 14,000 genes) into MCF-10A, a nontransformed breast cell line (12). Upon integration of the lentiviruses into the genome via puromycin selection, the resulting mixture of cells was grown for 5 d in flasks either with high-attachment or low-attachment surfaces. Representation of individual expressed genes in the resulting cell populations was determined via the barcodes. In principle, lentiviruses expressing oncogenes should yield preferential representation in the low-attachment vs. high-attachment populations.

Two observations validate both GILA and the genetic screen. First, the top hits are the well-known oncogenes H-Ras, K-Ras, and EGFR. Second, kinases, receptors, and signaling pathways linked to cancer are identified by gene-set enrichment analysis of the entire dataset based on rank-ordered barcode ratios from low:high attachment conditions (Fig. 5*A* and Fig. S6). In contrast, no gene sets are enriched when the genes of the dataset are ranked in inverse (high:low attachment) order.

To individually examine the oncogenic effect of individual genes more directly, MCF-10A cells were infected individually with lentiviruses overexpressing 62 candidate genes from the genetic screen, and the resulting stable cell lines tested for growth in high- and low-attachment conditions (Fig. 5*B*). As expected, cell lines overexpressing H-Ras, K-Ras, or EGFR grow much better in low- but not high-attachment conditions than the parental cell line, confirming their oncogenic properties. In addition, cells overexpressing MRPL20 or AKT2 grow preferentially in low- vs. high-attachment conditions ($P < 0.001$; Fig. S7); a few other genes (MAP3K3, EIF4E, PPP1R8, and C3orf62) may behave similarly ($P \sim 0.02$). Although not previously characterized as an oncogene, MRPL20 expression levels are part of a 16-gene principle components predictive of breast cancer risk (13). Although differences between growth in low vs. high attachment are modest for these genes, the results suggest that at least of some of them (and perhaps others implicated in the gene-set analysis) can make minor contributions to the oncogenic state. By analogy, deep sequencing on many cancer genomes reveals

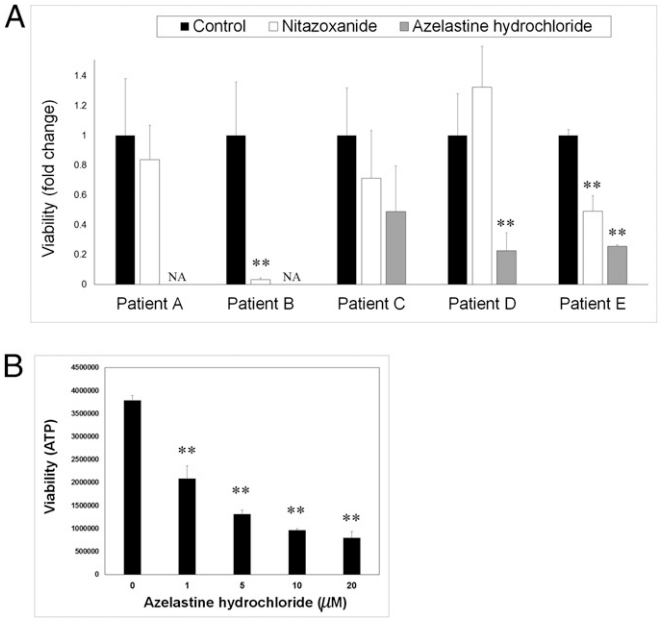

Fig. 4. Ex vivo drug sensitivity. (*A*) Representative results of drug activity of compounds established in initial drug screen in ex vivo GILA of ovary cancer patient-derived cells. Azelestine hydrochloride was found to be highly effective at inhibiting growth of these cells. (*B*) Azelestine hydrochloride concentration-dependent viability of ovary cancer patient-derived cells.

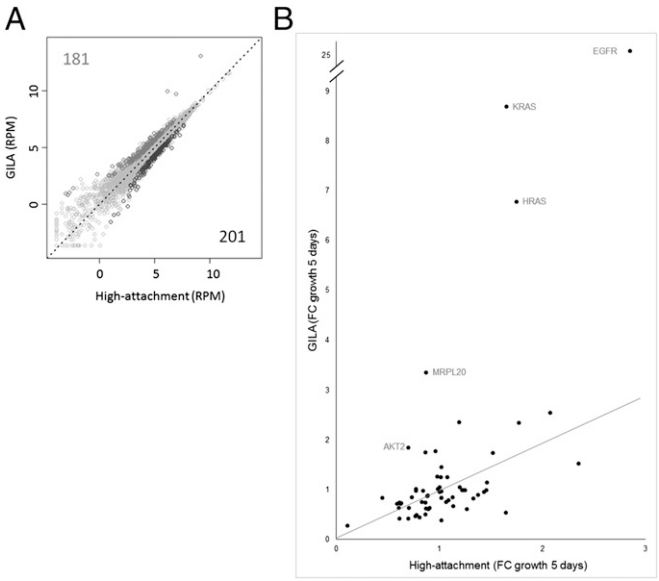

Fig. 5. Genetic screen for ORFs with oncogenic role in MCF-10A cells. (*A*) Overrepresentation of 181 barcoded ORFs under low attachment (dark gray shaded region on left) or 201 ORFs under high-attachment (black shaded region on right) conditions. (*B*) Validation of chosen ORFs by growth on low- and high-attachment surfaces. The ORFs show an increased (>1) growth in low attachment over time and a preferred growth in low attachment over growth on high-attachment surfaces (value of GILA/High-attachment ratio larger than 1). Values are fold changes of growth at day 0 to day 5.

many cancer-promoting or cancer-suppressing genes that individually make a minor contribution.

Discussion

GILA as a Replacement for the Soft-Agar Assay. The ability of cells to grow in soft agar is the gold standard and defining assay for cellular transformation that has been in routine use for decades. In principle, the GILA assay, which also requires cells to grow in an anchorage-independent manner under conditions of low attachment, should be similar to the soft-agar assay. By analyzing a variety of developmentally different cell lines, we show that the GILA assay is comparable, both qualitatively and quantitatively, to the classic soft-agar assay. We cannot exclude the possibility that these assays might give different results in other cell or cancer types, but these results are likely to be subtle because both assays are essentially measuring the same property of cell growth. We note that hematopoietic cells might not be suitable for GILA, because they do not require attachment for cell growth. Compared with the classical soft-agar assay, GILA is much faster (5 d instead of 3 wk), much less labor-intensive (essentially no work beyond seeding cells into wells), more practical (takes up less space in tissue culture incubators), more quantitative, and easier to score by using conventional plate readers. For these reasons, we strongly believe that GILA can replace the soft-agar assay to monitor cellular transformation.

Conceptually, it is useful and common in the cancer field to consider cells to exist in two distinct states, nontransformed or transformed, with these states being determined experimentally by the soft-agar assay. In reality, cellular transformation and cancer is not a single cellular state, but rather encompasses a continuum of phenotypes between the extremes of nontransformed and transformed states. The quantitative nature of the GILA assay is useful in this regard, because transformed cells can vary significantly in how well they grow on low-attachment conditions. Thus, the GILA assay can measure the degree of transformation for cell lines subjected to experimental perturbations on a population basis, something that is more difficult and more arbitrary to do with the soft-agar assay. However, the soft-agar assay, which measures colony formation from individual cells, is better equipped to analyze heterogeneity in the cell line, and in this regard, only a small percentage of cells in a typical transformed cell lines are capable of colony formation.

GILA for High-Throughput Drug and Genetic Screens. In addition to its advantages over the standard soft-agar assay for analyzing a limited number of cell lines and experimental perturbations, the GILA assay is suitable for high-throughput drug or genetic screens. Unlike screens relying on growth of cancer cells per se, GILA conditions are more specific and relevant to the transformed state, because they depend on a property of cancer cells that is not shared by normal or nontransformed cells. Furthermore, the combination of a GILA-based screen with a secondary screen that measures growth under conditions of attachment offers additional advantages. For example, drugs that inhibit growth in the GILA assay but not under standard conditions would be missed by a conventional screen, yet they are of potential interest as anticancer agents. Conversely, drugs that inhibit growth under both conditions may just be generally toxic to cells, although such drugs could still be valuable as potential therapeutic targets if they don't inhibit the growth of normal cells.

For genetic screens, it is of particular interest to identify genes that specifically inhibit or stimulate transformation in a manner that is distinct from cellular proliferation. However, genes that increase growth under both low- and high-attachment conditions may also be relevant for cancer. In addition to identifying known oncogenes (H-Ras, K-Ras, and EGFR) and unexpected genes (MRPL20), an advantage of the genetic screen performed here is the ability to identify candidate genes that may make minor contributions to the transformed state. As such, this approach complements large-scale sequencing of cancer genomes that identifies genes that make minor contributions to human cancer. Coherent groups of genes making minor contributions can be identified through gene-set enrichment analysis, and these genes are likely to reflect important pathways involved in the transformed or nontransformed states. One disadvantage of the GILA assay is that it is difficult to select rare transformed cells from a population of nontransformed cells. In contrast, colonies arising from individual rare transformed cells can be selected by using the soft-agar assay.

The GILA Assay in the Context of Personalized Medicine. Because every cancer is genetically, epigenetically, and phenotypically distinct, it is now well recognized that cancer treatment needs to be personalized. Furthermore, many drugs in clinical use for other diseases (e.g., diabetes, various inflammatory conditions, heart disease) have anticancer effects in vitro (8) and, hence, have the potential to be repurposed for treating cancer patients. For example, epidemiological data and preclinical experiments suggest the use of diabetes drug metformin for cancer prevention and treatment in nondiabetics (14–16), and clinical trials are in progress. For these reasons, we have initiated a phenotypic approach to personalized medicine in which patient-derived tumor cells will be screened for their response to a variety of FDA-approved drugs. In principle, FDA-approved drugs that inhibit specific cancer cells from a patient could be used off-label, individually or in combination, to treat that patient. This phenotypic approach is complementary to a genetic approach that uses DNA sequencing of a patient sample to identify putative oncogenes that confer sensitivity to drugs designed to specifically inhibit the identified oncoprotein.

The GILA assay is ideal for this personalized medicine approach, and as a proof of principle, we used it to examine cells from patients with ovarian cancer that did not respond to conventional chemotherapeutic treatment. We identified distinct

drug sensitivities in different patients in this limited study, and it should be straightforward to analyze most FDA-approved drugs (~500). Once potential drugs are identified, it would also be straightforward to examine the dose–response as well as combinatorial effects.

For this approach, it is critical that patient-derived cancer cells survive (and potentially grow) long enough for drug sensitivity to be assayed. In this regard, the GILA assay can be performed in a few days immediately after isolation from the patient. However, many cancer cells isolated from patients survive poorly outside the human body, so it is likely that some human cancers will not be amenable to this approach. For this reason, extending this approach to other cancer types may require experimental modifications (e.g., growth conditions, number of cells, length of incubation) that can only be addressed empirically. In addition, tumor samples invariably contain normal cells that may complicate the analysis, particularly if they survive or even grow better than the malignant cells from the tumor. This problem can be addressed by enriching for the tumor cells (typically by cell sorting) and/or measuring specific markers instead of ATP, but again the details and success will vary among different cancer types. Nevertheless, our limited experiments clearly show the potential of this approach, and it is likely that it will be useful for some, and perhaps many, forms of human cancer.

Materials and Methods

Cell Culture. The nontransformed breast cell line MCF-10A (12) was grown in DMEM/F12 medium supplemented with 5% (vol/vol) donor horse serum, 20 ng/mL epidermal growth factor, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, and antibiotics (penicillin/streptomycin) (17). Other breast cell lines (T47D, MDA-MB-231, and MDA-MB-486) were grown in DMEM media, 10% (vol/vol) FBS, and antibiotics. The three BJ fibroblast lines (EH, EL, ELR) were cultured in KO-DMEM media, 15% (vol/vol) FBS, 16.5% (vol/vol) Medium 199, 3.5 mM L-glutamine, and antibiotics (9). Nontransformed WI-38 lung fibroblasts were grown in Eagle's MEM supplemented with 10% (vol/vol) FBS and antibiotics. Not transformed (BPH1) and transformed (PC3) prostate cell lines were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) FBS and antibiotics (18). Three subcloned cell lines from human ovarian cancer primary cells were maintained in KO-DMEM media, 15% (vol/vol) FBS, 16.5% (vol/vol) Medium 199, 3.5 mM L-glutamine, and antibiotics. SAA1 is a cell line with a low-grade transformation value, SAA2 is a mild-grade, and SAA3 has the highest transformation value, as assessed in vitro and in vivo. Unless otherwise mentioned (*Acknowledgments*), cells were purchased from the American Type Culture Collection. All cells were grown in a 5% CO_2-humidified incubator at 37 °C.

Soft-Agar Assays. Cells (10^5 per well) were mixed with 0.4% agarose in growth medium, plated on top of a solidified layer of 0.5% agarose in growth medium, in a 24-well plate, and fed every 3 d with growth medium. After 3–4 wk, the colonies were dyed with Cristal Violet (0.01% solution), washed with PBS, and imaged by using a custom, automated plate imager with a digital camera (Olympus SP-350; Cam2Com) (19). A custom MATLAB program was developed to first detect each well in the plate, based on Canny edge detection and morphological post processing. Colonies were detected within each well by using the wavelet transform-based detection algorithm (20). Image pixel sizes were calibrated by relating user input of the physical size of the plate to the detected spacing between wells in image space. This calibration was used to calculate colony area in square microns and to calculate equivalent radii. This calculation produced both net colony density for each well.

GILA Assay and Drug Screens. Transformed fibroblast cells were maintained as subconfluent monolayers, trypsinized, and seeded in 96-well plates (for analytical assays; Fig. 1) or in 384-well white plates (for screens; Fig. 3). For many experiments, the cell concentration was optimized to 1,000 cells per well in 100 μL of medium (for 96-well plate) or 50 cells per well in 30 μL of growth medium (for 384-well plate). However, other applications such as growth of cells from patient tumors or alternative growth media may require optimization of cell number and assay time. Two types of plates were used in the screens: high-attachment conditions (Corning; 3704) and Ultra Low-attachment (Sumitomo; PrimeSurface384U). Cells were seeded automatically by using the liquid rapid dispenser Metrix WellMate (Thermo). Two small molecule libraries (NIH Clinical Collection 1–2013 and Kinase Inhibitor Focused Library) were transferred to each microplate, at 100 nL of drug per well, to a final concentration of 30 μM or 10 μM, respectively, by using the robotic transfer system (Seiko Epson) and pin array (V&P). Controls were added by multichannel pipette. After 5 d of incubation at 37 °C in 5% CO_2, the cells were assayed for ATP content. Each plate had a duplicate, and the entire screen was repeated two times. The secondary screen was conducted similarly, except that an HP D300 Digital Liquid Dispenser and T8 dispense-heads (HP) were used to add four different concentrations of small molecules to the cells.

Cell Viability Assay. Cell viability was measured with the CellTiter-Glo (Promega) luminescent assay, using the EnVision Plate Reader (PerkinElmer) or the SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices). For high-attachment growth, tissue culture-treated 96-well clear plates (3997; Corning) were used, whereas for the GILA assay, low-attachment surface plates (3474; Corning) of 96-well clear plates were used for 3–5 d. Later, the cells were moved to 96-Well Solid White Polystyrene Microplate (3362; Corning) for reading luminescence signal. To avoid bleeding of luminescence between the wells, every other well was left empty in the solid white microplate. Thirty microliters of CTG reagent was used for 384-well microplates, and 100 μL for 96-well microplates. The cells were moderately shaken for 5 min in the reagent, followed by incubation for 20 min to ensure cell lysis. No background signal was detected from wells containing only culture medium.

Identification of Hits from the Drug Screen. Cross-talk corrected values from EnVision reader were used to normalize the luminescence from each wells. Negative control values were used to calculate the Z score for each well in each replicate, with the formula (Luminesence – average of negative control)/SD of negative control. Z score values were graded strong (S), moderate (M), or weak (W), where $-5 > S$, $-5 > M > -4.5$, and $-4.5 > W > -3.2$. To be considered a hit, a drug had to score positive in one of replicates on the low-attachment plates and to score negative in the high-attachment condition. Finally, the selected hits had to have a ratio of luminesce (low/high attachment) <0.25.

Patient Samples. Under an institutional review board approved protocol, freshly discarded ascites from patients with advanced stage ovarian cancers was collected from five individuals. Ascites fluid was immediately transferred on ice for further processing. Samples were spun down, and red blood cells were removed with hypotonic lysis by using ACK lysing buffer (Life Technologies). Cells were filtered through a 40-μm mesh and washed in PBS with 2% (vol/vol) FBS and subjected to staining for FACS.

Flow Cytometry. Cells were prepared per standard protocols, stained with Calcein-AM, CD45-FITC, EPCAM-PE, and CD24-PE/Cy7, and incubated for 20 min on ice. Cells were washed with FBS with 2% (vol/vol) FBS before flow sorting. After doublet discrimination and compensation for spectral overlap, single, viable cells, negative for CD45 and positive for EPCAM and CD24, were flow-sorted into wells (96-well plates) and prepared with growth medium. Samples were sorted on a BD FACSAria SORP and analyzed by using BD FACSDiva Software (BD Biosciences).

Oncogenic ORF Screen. MCF-10A cells were infected with a library of barcoded ORFs (21) at a low (<1) multiplicity, with an average of 1,000 cells per ORF clone, and infected cells were selected with puromycin for 5 d. Genomic DNA samples were taken from the resulting cells and noted as early time point (ETP). These cells were then seeded into two types of flasks with different surfaces—traditional high attachment (430641; Corning) or ultralow attachment (3814; Corning) and grown for an additional 5 d. Following genomic DNA preparation, PCR was performed to amplify the barcode, followed by Illumina sequencing of the product to determine the relative abundance of each ORF in the different attachment conditions. Genes of interest were identified as having significantly different representation (by sequence reads) from libraries from the low-attachment condition as opposed to the high-attachment condition.

A secondary validation screen was performed on 62 genes selected from the primary screen by using the following criteria: values of GILA over high-attachment growth > 1.5 fold; expression values consistently up-regulated > 1.2-fold and Poisson test P value < 1e−4 (~ Benjamini Hochberg corrected FDR 0.01). We furthermore required that the ORF-encoding genes should be well expressed in breast tissues, using RNA-sequencing data from the Cancer Genome Atlas database (22). For each gene to be tested, the appropriate lentivirus expressing this gene was infected into MCF-10A cells, and infected cells were selected with puromycin for 5 d. The resulting cells were tested in 96-well low- and high-attachment plates and assessed by

the cell viability assay described above. Statistical significance for oncongenic behavior of individual genes was determined with respect to the normalized distribution of tested targets centered on a trimmed mean, a conservative approach.

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