Published in final edited form as:

Cancer Metastasis Rev. 2018 December ; 37(4): 655–663. doi:10.1007/s10555-018-9767-4.

Tackling tumor heterogeneity and phenotypic plasticity in cancer precision medicine: our experience and a literature review

Shijie Sheng^{1,2,3}, M. Margarida Bernardo^{1,3}, Sijana H. Dzinic^{2,3,4}, Kang Chen^{2,4,5}, Elisabeth I. Heath^{2,4}, Wael A. Sakr^{2,3}

¹Department of Pathology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, MI 48201, USA

²Department of Oncology, Wayne State University School of Medicine, Detroit, MI 48201, USA

³Tumor Biology and Microenvironment Program, Wayne State University School of Medicine, Detroit, MI 48201, USA

⁴Molecular Therapeutics Program of the Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201, USA

⁵Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI 48201, USA

Abstract

The predominant cause of cancer mortality is metastasis. The major impediment to cancer cure is the intrinsic or acquired resistance to currently available therapies. Cancer is heterogeneous at the genetic, epigenetic, and metabolic levels. And, while a molecular-targeted drug may be pathway-precise, it can still fail to achieve wholesome cancer-precise toxicity. In the current review, we discuss the strategic differences between targeting the strengths of cancer cells in phenotypic plasticity and heterogeneity and targeting shared vulnerabilities of cancer cells such as the compromised integrity of membranous organelles. To better recapitulate subpopulations of cancer cells in different phenotypic and functional states, we developed a schematic combination of 2-dimensional culture (2D), 3-dimensional culture in collagen I (3D), and mammosphere culture for stem cells (mammosphere), designated as Scheme 2D/3D/mammosphere. We investigated how the tumor suppressor maspin may limit carcinoma cell plasticity and affect their context-dependent response to drugs of different mechanisms including docetaxel, histone deacetylase (HDAC) inhibitor MS-275, and ionophore antibiotic salinomycin. We showed that tumor cell phenotypic plasticity is not an exclusive attribute to cancer stem cells. Nonetheless, three subpopulations of prostate cancer cells, enriched through Scheme 2D/3D/mammosphere, show qualitatively different drug responses. Interestingly, salinomycin was the only drug that effectively killed all three cancer cell subpopulations, irrespective of their capacity of stemness. Further, Scheme 2D/3D/mammosphere may be a useful model to accelerate the screening for curative cancer drugs while avoiding costly characterization of compounds that may have only selective toxicity to some, but not all, cancer cell subpopulations.

Shijie Sheng, ssheng@med.wayne.edu.

Conflict of interest The authors declare that they have no conflicts of interest

Keywords

Drug resistance; Heterogeneity; Cancer stem cells; Proliferation; Transient quiescence; Epithelial-to-mesenchymal transition (EMT); Integrity of membranous subcellular structures; Drug screening strategy; Cell death; Cell survival; Cancer strength; Cancer vulnerability; Maspin; Scheme 2D/3D/mammosphere; Docetaxel; MS-275; Salinomycin

1 Introduction

According to a projection by the American Cancer Society (ACS), more than 1,700,000 new cancer cases will occur in the USA in 2018 [1]. Current approaches to cancer management include early diagnosis and cancer-specific treatment modalities such as surgery, radiotherapy, and chemotherapy. Unfortunately, more than 600,000 Americans are projected to die of cancer in 2018. Based on the record of the World Health Organization, cancer is the second leading cause of death, responsible for about one in six deaths in the world [2]. A major impediment to a cancer cure is the intrinsic or acquired resistance to currently available therapies [3–5]. Advancements in mechanistic studies of cancer biology have led to the identification of numerous potential therapeutic targets [6]. Robust combinatorial chemistry has revolutionized the possibility and efficiency of making compounds of almost unlimited structural varieties [7, 8]. With the initiatives of the National Cancer Institute, well-characterized cancer and normal control cell lines can be tested simultaneously to minimize cell line-associated artifacts in drug screening [9]. The validation of potential therapeutic agents can be facilitated by an increasing number of animal tumor models, including patient-derived xenografts (PDX), cell line-based xenograft models [10], genetically modified animals [11, 12], as well as *in vitro* cell culture models [13, 14]. The measurements of drug-induced cellular phenotype changes have incorporated automation of multiplex *in vitro* format, for detection of a large number of biological and molecular endpoints, and high-resolution imaging modalities for quantification of *in vivo* tumor formation [15]. However, a major hurdle in treating cancer continues to lie in our inability to overcome its heterogeneity at multiple levels. Current drug screening strategies do not adequately recapitulate the complexity of drug response by subpopulations of cancer cells of diverse phenotypes. To date, we are yet to develop curative chemotherapeutics that eliminate not only the bulk of the tumor but also the drug-resistant residual cancer cells.

2 Cancer cell elimination as the goal of precision Cancer medicine

The predominant cause of cancer mortality is tumor metastasis, leading to tumor colonization and destruction of additional organs of selective preference. As summarized by Hanahan and Weinberg [16], tumor metastasis results from the continuum of cross-talk between tumor cells and their host environments, which normally impose barriers (e.g., immune surveillance) and serious threats (e.g., cell death signals) to tumor cells. To successfully complete the metastatic process, cancer cells undergo multi-directional morphological transformations from a normal epithelial architecture to pliable phenotypes including, but not limited to, epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET). Inadvertently, as summarized in Fig. 1a, the gain of functional attributes that enables cancer cell immortality, survival, and phenotypic

plasticity is at the cost of normal cell integrity. For example, tumor cell transformation results from the loss of genetic integrity, cell cycle checkpoints, and the control of proliferation by cell-cell and cell-matrix contacts. The invasiveness of cancer cells is propelled by the lack of fine-tuned stromal wound healing and reduced dependence of tumor cells on appropriate and intact extracellular matrix. For cancer cells to evade host immune surveillance, they can curtail the immunogenic “danger” alert and induce immune repression. It is of paramount importance to keep in mind that it is the tumor cells that spread from the organ/tissue of origin to distant sites. Thus, cancer cells are the ultimate driving force in tumor metastasis, and must be the ultimate drug target. That is why surgical removal of anatomically confined early stage tumor can be curative, whereas chemotherapeutic treatments that fail to completely eradicate residual tumor cells inevitably lead to drug-resistance and recurrence.

Overwhelming evidence demonstrates that cancerous lesions are always composed of heterogeneous cell populations of mixed phenotypes. Targeting phenotypic plasticity is critically important in case it underlies the acquired resistance of cells to death or promotes sustained survival [17]. If the therapeutics only block the phenotypic plasticity but stop short of eliminating the tumor cells, the treatment will not be curative. On the other hand, any treatment that eradicates only a subpopulation of cancer cells will leave the door open for recurrence. The fundamental concept of precision medicine is inspired by the possibility of personalized strategy to target cancer patients according to their cancer genetics [18, 19]. However, while DNA mutations and genetic instability lie at the heart of cancer development and progression, genetic changes alone, which are irreversible, cannot adequately explain the phenotypic plasticity cancer cells manifest throughout metastasis. Mutations in genes such as the Her2/neu oncogene and p53 tumor suppressor do not occur in all tumor cells and/or concomitantly in tumor progression [20, 21]. The probability of eliminating all viable tumor cells by targeting a particular cancer-driving genetic mutation is low. Thus, despite the exciting developments of molecular-targeted therapeutics with significant survival benefits to patients, these cancer genetics-based therapeutic strategies are not curative cancer-specific precision medicine.

Arguably, the best example for the ideal curative precision cancer medicine for metastatic cancer is the targeted immunotherapy that can elicit fatal toxicity to tumor cells while sparing the integrity and functions of normal cells in the hosts. For example, checkpoint immunotherapy that targets the immune repression mechanism mediated by tumor-derived programmed cell death protein 1 (PD-1) has achieved the breakthrough of long-term remission of metastatic cancer of various organs of origin and is generally well-tolerated [22]. The prostate cancer-specific autologous vaccine Sipuleucel-T, which is patients’ dendritic cells stimulated by a fusion protein consisting of prostatic acid phosphatase and granulocyte-macrophage colony stimulating factor, is curative in treating some cases of asymptomatic minimally metastatic prostate cancer [23]. The probability for the totality of immunotherapy-elicited cancer elimination attests that at least some tumor cells have acquired antigenicity that can be recognized by the host immune surveillance as danger alerts. On the other hand, host immunity against cancer, once activated, has the unparalleled advantage in its adaptive nature, persistent memory, and the intricate endogenous control of the aptitudes of the cytotoxic effects. Unfortunately, in checkpoint immunotherapy, tumor

expression of programmed death-ligand 1 (PD-L1) does not reliably predict a patient's response [22]. Similarly, there is no clear rationale to predict the prostate cancer patients' response to Sipuleucel-T [23]. It is reasonable to believe that future breakthroughs in cancer immunotherapy depend on better strategies and methods to identify immune-evasive cancer subpopulations.

3 The challenges of insufficiency in targeting the “strengths” of cancer cells

The gain of functions, as summarized in Fig. 1b, adds profound strengths to propel metastatic cancer cells through tumor progression and resist death threats. Compared to normal cells, cancer cells exhibit a higher order of freedom in their genetics and metabolic functions [24, 25]. Extensive experimental evidence demonstrates that manipulation or perturbation of any of the molecular pathways in cancer cells leads to cascades of not only direct responses but also indirect changes, ultimately elevating cellular defenses to counteract the initial changes. For example, despite the promising preclinical evidence that targeting a node of signal transduction by inhibiting the protein chaperone heat shock protein 90 (Hsp90) [26], clinical trials of multiple generations of Hsp90 inhibitors were not effective in improving progression-free survival and overall survival of cancer patients [27, 28]. In addition, pharmacological inhibitors of the PI3K/AKT/mTOR oncogenic signaling pathway actually enhance cellular survival, at least in part, through autophagy [29–31].

A critical gain of function of cancer cells is their independence from the confined communal cell-cell organization for synchronized normal epithelial cells [32, 33]. Although cancer cells actively communicate among themselves, with stromal cells, and with acellular components in the tumor microenvironment, they can autonomously enter cell cycle, remain quiescent, undergo EMT, carry out basement membrane (BM) and extracellular matrix (ECM) invasion, or undergo MET. At any given time, if chemotherapy targets the cell cycle machinery, quiescent cancer cells may be spared. On the other hand, proliferating cancer cells may or may not be sensitive to drugs that target the underlying epigenetic control for EMT or MET such as HDAC inhibitors [34] or TGF- β inhibitors [35, 36].

The difficulty to devise indiscriminate cancer precision medicine may be alleviated if we can identify the blueprints of all malignant traits. To this end, the concept of cancer stem cells (CSC) offers a reasonable framework to investigate the origin, progression, and drug resistance of cancer [37–40]. CSCs are typically a subpopulation of cancer cells that can experimentally proliferate in self-renewal mode in the special mammosphere suspension culture. Most of the CSCs have been shown to express various combinations of molecular markers that are shared by normal stem cells. As compared to normal stem cells that are immortal and capable of spatially and temporally-controlled self-renewal and lineage-directed differentiation for the biogenesis of tissues and organs, the self-renewal of CSCs is not under developmental programmatic controls.

Further, the “differentiation” of CSCs seems to have neither lineage specificity nor apparent terminal point. Since CSCs are associated with increased tumorigenicity and drugresistance *in vitro* and in xenograft tumor models, they may be the very cell population giving rise to

cancer cell phenotypic plasticity. If that is the case, CSCs should be the ultimate autonomous engine that sustains tumor progression, metastasis, and drug resistance. Therefore, CSCs could be the ideal drug target for curative cancer treatment.

To date, the CSCs-based approach is yet to prove its potential to indiscriminately kill all cancer cells and prevent cancer recurrence *in vivo*. There are several major obstacles to the application of the CSCs concept. First, the immortality of cancer cells is not reserved to only CSCs as patient-derived xenograft with heterogeneous tumor cell populations or established cancer cell lines, of which only a small percentage bears the traits of CSCs, can be passed continuously under appropriate conditions. Second, it is yet to be established that non-CSC tumor cells cannot proliferate or undergo differentiation to any extent. Third, there is yet evidence to show that the drug-resistance mechanisms in CSCs are not shared at all by the non-CSC tumor cells. In addition, it is yet to be established that a single or multiple CSC populations are responsible for cancer heterogeneity.

4 Opportunities of targeting cancer cell “vulnerabilities”

The gains of cancer cellular functions and survival strengths also shift the epithelial homeostasis away from integrity, which may render cancer cells vulnerable. If cancer cells share some common points of vulnerability, targeting those vulnerabilities may kill cancer cells regardless of their heterogeneity and phenotypic plasticity. It is common knowledge that when DNA is damaged, cells die. Cancer cells are more vulnerable than normal cells to DNA-damaging agents because they are more proliferative and their DNA is more exposed. It is also well-established that the dynamic microtubule polymerization and depolymerization is essential for mitosis. For this reason, cancer cells are more sensitive than normal cells to microtubule-targeted drugs such as taxotere. Unfortunately, even when these drugs are used in combination for improved efficacy, only a subset of patients is sensitive to the treatment and survival benefits are limited.

Cell death can also be elicited when the plasma membrane and/or membrane-encapsulated organelles are irreversibly damaged [41, 42]. Furthermore, it has been demonstrated that the membranous organelles are integrated to sustain cell survival and stress response. Conversely, damage to organelle membrane integrity can directly elicit cell death. For example, mitochondrial membrane permeability is the most important switch of irreversible apoptosis. Lysosomes contain a large number of catabolic hydrolases that contribute to autophagy-mediated cell death. Stressed endoplasmic reticulum (ER) causes the accumulation of unfolded proteins in the ER lumen, unfolded protein response, and perturbations of steady-state levels of calcium, all of which can trigger cell death.

For cancer cells to survive and undergo phenotypic changes, they are constantly stressed to regenerate housekeeping subcellular structures including membranous organelles. As a result, the integrity of the plasma membranous or membranous organelles may be commonly compromised. It has been shown that cancer cells are iron-seeking, which make them vulnerable to drug-induced ferroptosis [43], a subtype of necrosis that features unchecked lipid peroxidation and consequent collapse of the lipid bilayer membrane [44, 45]. Direct triggers of ferroptosis include iron-dependent production of reactive oxygen species (ROS)

and inactivation of glutathione peroxidase 4 [46]. Alternatively, ferroptotic cell death can be induced by cytoplasmic depletion of iron by ionophores, such as polyether antibiotic salinomycin [47] or its derivatives, which, in turn, leads to iron loading in lysosomes [48].

Our group showed, in 2015, that salinomycin is highly toxic to prostate cancer cells, including CSCs and drug resistant cancer cell lines, without any significant toxicity to normal prostatic epithelial cells [49]. These results are consistent with other reports [47] [1]. Recently, similar results were reported with a synthetic derivative of salinomycin, AM5, in a study of breast cancer cells and breast CSCs [48]. Consistent with the notion that cancer cells exhibit increased cross-talk among membranous organelles, salinomycin also enhances the permeability of mitochondria [50] and vacuoles [51]. Thus, lipid bilayer-targeted drugs may be novel and promising chemotherapeutic agents, irrespective of cancer types, cancer cellular phenotypes, and the level of cancer cell stemness.

5 A strategic approach to recapitulate cancer cell phenotypic heterogeneity

A bottleneck in the discovery of curative cancer-targeted precision medicine continues to be the lack of a comprehensive strategy to fully assess the differential drug sensitivity derived from cancer phenotypic heterogeneity. Cancer cells are distinct from normal cells for their overall freedom of phenotypic directionality in their response to oncogenic pressures in various microenvironments [52–54]. In the same tumor tissue, cancer cells with different balances between oncogenic and tumor suppressive operatives may derive distinct phenotypes under the same internal and external pressures. However, we propose that regardless of the versatile underlying mechanisms, the proliferative states of cancer cells may be switched among three different modes: exponential proliferation, transient quiescence, and CSC-specific self-renewal. We postulate further that these three different cellular processes may dictate different responses to DNA-targeted drugs and inhibitors of non-genomic mechanisms. In addition, regardless of the differentiation grades or proliferative status, the perpetual tumor progression tends to compromise the integrity of lipid bilayer membranes, thus elevating tumor sensitivity to drugs that attack membranous structures and functions.

Our team developed a schematic combination of cell culture cancer models, designated as Scheme 2D/3D/mammosphere [49], based on the following considerations. The commonly used monolayer (2D) maintenance culture supports a combination of surviving cells and cells undergoing exponential growth. If the tumor mass contains CSCs, this small cell population can be serially enriched by mammosphere suspension culture (mammosphere), wherein CSCs are supported for self-renewal (or symmetric division). The 3D culture in collagen I, the most abundant substratum in ECM, has been shown to support epigenetic reprogramming for various cellular organizations and phenotypes [55]. As cancer cells transition among various states of differentiation in 3D COL culture, they may be restricted in cell proliferation or undergo quiescence. As schematically illustrated in Fig. 2a, we showed that in 2D cultures, poorly differentiated cell lines of prostate cancer such as DU145, PC3 and LNCaP C4–2B, and mouse cell lines derived from the PTEN conditional

knockout model for prostate cancer are highly invasive and exhibit EMT hallmark gene expression profiles. When single cell suspensions prepared from the 2D cultures of these cells were grown in 3D COL culture, these cells formed solitary and solidly packed tumor nodules with the peripheral cells infiltrating the collagen matrix. A small percentage in each of these cell lines exhibited CSC characteristics *in vitro* and was highly tumorigenic *in vivo* [49]. The CSCs, when grown in 3D COL culture formed solitary and solidly packed tumor nodules at higher frequencies than the corresponding cells harvested directly from the 2D culture.

It is well established that *in vivo* malignant tumors, such as prostate cancer, are typically a heterogeneous mixture of cells of various differentiation grades at any given point and become increasingly more heterogeneous with disease progression. We have been particularly interested in how the tumor suppressor maspin [56] may limit carcinoma cell phenotypic plasticity and affect the context-dependent cancer cell response to drugs of different mechanisms. Maspin gene deletion in germ cells aborts embryogenesis [57]. In somatic cells, maspin expression is epithelial-specific [58–60]. Conditional deletion of the mouse maspin gene leads to an array of epithelial abnormalities, including grade 2–3 lung adenocarcinoma, mammary gland myoepithelial hyperplasia in female mice, and dorsal lateral prostate hyperplasia in male mice [57]. At the molecular level, maspin is an endogenous epithelial-specific inhibitor of HDAC1 [61], and consequently controls the expression of a cluster of genes functionally involved in cell differentiation [49, 55]. In clinical specimens of various types of carcinomas, maspin expression inversely correlated with tumor progression [62]. Further, maspin is found to be severely downregulated in established cancer cell lines. Maspin transfection of cancer cell lines leads to reversal of tumor cell EMT, invasion, and metastasis, without directly regulating cell proliferation [56, 63–65]. In particular, maspin-transfected DU145 cells grew as better-differentiated epithelial-like acinar structures, in contrast to the solid tumor sheets of the mock transfected DU145 cells. As illustrated in Fig. 2b, when cultured in 3D COL, maspin-transfected cells formed epithelial-like acini, but failed to form CSC colonies in the mammosphere assay. Interestingly, maspin-transfected cells survived as aggregates, which failed to form tumor colonies in 3D COL culture, and lost their tumorigenicity *in vivo*. The cellular aggregates of the maspin-transfected cells in the mammosphere assay continued to survive for up to 6–7 passages in the mammosphere assay, demonstrating an increase in autophagy-induced survival, and eventually died of senescence [49].

Clearly, the traditional 2D adherent culture system is insufficient to recapitulate the heterogeneous cancer phenotypes. In our hands, experimental control of maspin expression in prostate tumor cells in the schematic 2D/3D/mammosphere culture system demonstrates the capacity of tumor cells to exist in multiple distinct differentiation states including: CSCs in self-renewal mode under challenging microenvironments, tumor mass capable of invading the stroma, better differentiated phenotype with a higher level of preservation of epithelial-like cellular organization, and better differentiated cells in autophagy survival mode under extremely challenging conditions where only CSC-like cells survive. Importantly, the phenotypic plasticity or phenotypic heterogeneity is not an exclusive attribute to CSCs. As demonstrated by our study of maspin, genetically similar non-CSC tumor cells may display various levels of phenotypic plasticity in a context-dependent manner.

6 Scheme 2D/3D/mammosphere for cancer-targeted precision medicine screening

The phenotypic segregation by the 2D/3D/mammosphere culture scheme raised the hope that this scheme may allow the identification of therapeutic agents that can exert cytotoxicity with similar efficiency to the full spectrum of phenotypic variations in heterogeneous tumor masses [49]. We tested this idea by treating isogenic pairs of prostate cancer cells derived from DU145 and PC3. In the case of DU145 cells that do not express endogenous maspin; maspin is expressed ectopically by a transgene. In the case of PC3 cells that still express endogenous maspin, maspin expression is knockdown by maspin-specific small hairpin RNA (shRNA).

Several noteworthy observations are summarized in Fig. 2c. First, different subpopulations of prostate cancer cells had different responses to the same drug. For example, docetaxel, currently used Several noteworthy observations are summarized in Fig. 2c. First, different subpopulations of prostate cancer cells had different responses to the same drug. For example, docetaxel, currently used abetter-differentiated phenotype is associated with decreased drug sensitivity to docetaxel. Although MS-275, a pharmacological inhibitor of class I HDACs, was toxic to cells in 2D and mammosphere cultures, it partially induced epithelial differentiation and lost the cytotoxicity in 3D COL culture. Third, maspin seems to exert different effects on different tumor cell subpopulations. On one hand, consistent with the notion that epithelial cells in polarized 3D acinar structure are in transient quiescence, the maspin-expressing tumor cells in 3D COL culture were less sensitive than the maspin-nonexpressing cells to docetaxel. On the other hand, the maspin-expressing tumor cells were more sensitive than maspin-nonexpressing tumor cells to MS-275 and salinomycin. These data are in line with our earlier observation that maspin does not directly regulate cell cycle progression but sensitizes exponentially growing tumor cells to drugs that directly induce cell death [64, 69–71]. Finally, salinomycin was the only drug that targeted all cell subpopulations, albeit with varying potencies. It is important to note that normal prostate epithelial cells that express a high level of maspin did not give rise to mammospheres or tumor colonies in 3D COL culture. These normal epithelial control cells are insensitive to the aforementioned treatments [49], supporting the tumor-specificity of the targets of these drugs, regardless of how insufficient they may be.

Interestingly, we noted that drugs with lower EC_{50} , the concentration of a drug that gives half-maximal response, were not necessarily more effective in achieving maximal cytotoxicity (E_{max}). For example, docetaxel in 2D culture had an EC_{50} in the nanomolar range. However, the concentrations at which maximum effect was achieved (E_{max}) plateaued at approximately 60%, thus revealing that 40% of the cells were drug resistant. It is conceivable that even in culture conditions that support exponential growth, some cells may not be in active symmetric proliferative cell cycle and may not be sensitive to drugs that target proliferation. For the same reason, it is not surprising that the enriched CSCs or cells that developed stable cellular organization (e.g., the maspin-expressing cells in 3D COL culture) were insensitive to docetaxel. In parallel, both maspin-expressing and –non-expressing cells in 3D COL culture were insensitive to MS-275, suggesting that ECM-

confined tumor cells, that have reduced capacity for further phenotypic plasticity or growth, may be less dependent on epigenetic reprogramming. Cells that are unrestricted either for proliferation or for phenotypic transition may generally need the freedom of epigenetic reprogramming, thus can be generally susceptible to MS-275. Consistent with this notion, although MS-275 displayed different EC_{50} values with maspin-expressing and maspin-non-expressing tumor cells, it had similar E_{max} in 2D and mammosphere cultures of all the cell lines tested. By far, salinomycin was the most effective among the three drugs tested in killing all the tumor cell subpopulations. This result underscores the general and critical membranous integrity vulnerability in all tumor cell subpopulations.

Taken together, Scheme 2D/3D/mammosphere may be used as a first line *in vitro* system to screen for drugs with the broadest spectrum of cytotoxicity against CSCs and other cancer cell subpopulations of distinct differentiation grades. Using this system, we showed that cancer drugs that target either a particular strength (e.g., the elevated epigenetic reprogramming) or a particular vulnerability (e.g. the necessity to complete the cell cycle) may not target all the cancer cells in a heterogeneous tumor cell population. In comparison, drugs that target lipid bilayer membranes may be advantageous to exert cytotoxicity to most, if not all, cancer cell subpopulations.

7 Concluding remarks

To develop curative cancer chemotherapies, we need to focus on the elimination of cancer cells, which are the source of all cancer-related pathologies including tumor cell stemness, context-dependent tumor phenotypic plasticity, inflammatory tumor microenvironment, and evasion of host immune surveillance. Currently, chemotherapy is provided to patients who are ineligible for curative regimens such as surgery, or who are unresponsive to potentially curative immunotherapies. Unfortunately, cancer is heterogeneous in multiple aspects. A molecular-targeted drug may be pathway-precise, but is likely not to achieve total tumor elimination. From a cancer death-focused perspective, it is important to target the strengths of cancer cell operatives such as genetic instability and increased freedom of epigenetic, metabolic, and redox circuitry reprogramming. However, targeting the vulnerability shared by all tumor cells in heterogeneous tumor masses, such as the increased need for biogenesis and function of membranous structure, may be a more cancer-specific approach to eradicate cancer. In order to facilitate the screening of curative cancer drugs, we need a robust cancer biology-based experimental system that recapitulates subpopulations of distinct phenotypes. Our study suggests that Scheme 2D/3D/mammosphere-based drug screening may provide a strong biological rationale for exclusion in subsequent *in vivo* validations using appropriate models. In Scheme 2D/3D/mammosphere, salinomycin exhibits a potential to overcome tumor cell heterogeneity and plasticity. While the clinical application of salinomycin needs to be investigated further, Scheme 2D/3D/mammosphere may have a broad application in preclinical studies to accelerate the development of curative cancer drugs, while reducing the cost of premature mechanistic studies and pharmacological characterization of compounds that may have only selective toxicity to some, but not all, cancer cell populations.

Financial support

This work was supported by the NIH Grant P30CA022453 (to the Karmanos Cancer Institute (KCI) with Sheng, S. as a program leader), the Ruth Sager Memorial Fund (to Sheng, S.), the KCI Pilot Project Grant 25S5Z (to Sheng, S.), the KCI Prostate Cancer Research Pilot Project Grant (to Sheng, S.), and the KCI Tumor Biology and Microenvironment Program Pilot Project (to Sheng, S).

References

1. Siegel RL, Miller KD, & Jemal A (2018). Cancer statistics, 2018. *CA: a Cancer Journal for Clinicians*, 68(1), 7–30. 10.3322/caac.21442. [PubMed: 29313949]
2. Global Burden of Disease Cancer, Fitzmaurice C, Akinyemiju TF, Al Lami FH, Alam T, Alizadeh-Navaei R, et al. (2018). Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability adjusted life-years for 29 cancer groups, 1990 to 2016: a systematic analysis for the global burden of disease study. *JAMA Oncology*. 10.1001/jamaoncol.2018.2706.
3. Holohan C, Van Schaeybroeck S, Longley DB, & Johnston PG (2013). Cancer drug resistance: an evolving paradigm. *Nature Reviews Cancer*, 13(10), 714–726. 10.1038/nrc3599. [PubMed: 24060863]
4. Maugeri-Sacca M, Vigneri P, & De Maria R (2011). Cancer stem cells and chemosensitivity. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, 17(15), 4942–4947. 10.1158/1078-0432.CCR-10-2538. [PubMed: 21622723]
5. McMillin DW, Negri JM, & Mitsiades CS (2013). The role of tumor-stromal interactions in modifying drug response: challenges and opportunities. *Nature Reviews. Drug Discovery*, 12(3), 217–228. 10.1038/nrd3870. [PubMed: 23449307]
6. Maertens O, McCurrach ME, Braun BS, De Raedt T, Epstein I, Huang TQ, et al. (2017). A collaborative model for accelerating the discovery and translation of cancer therapies. *Cancer Research*, 77(21), 5706–5711. 10.1158/0008-5472.CAN-17-1789. [PubMed: 28993414]
7. Plowright AT, Ottmann C, Arkin M, Auberson YP, Timmerman H, & Waldmann H (2017). Joining forces: the Chemical Biology-Medicinal Chemistry Continuum. *Cell Chemical Biology*, 24(9), 1058–1065. 10.1016/j.chembiol.2017.05.019. [PubMed: 28602761]
8. Liu R, Li X, & Lam KS (2017). Combinatorial chemistry in drug discovery. *Current Opinion in Chemical Biology*, 38, 117–126. 10.1016/j.cbpa.2017.03.017. [PubMed: 28494316]
9. Erickson BK, Rose CM, Braun CR, Erickson AR, Knott J, McAlister GC, et al. (2017). A strategy to combine sample multiplexing with targeted proteomics assays for high-throughput protein signature characterization. *Molecular Cell*, 65(2), 361–370. 10.1016/j.molcel.2016.12.005. [PubMed: 28065596]
10. Weeber F, Ooft SN, Dijkstra KK, & Voest EE (2017). Tumor organoids as a preclinical cancer model for drug discovery. *Cell Chemical Biology*, 24(9), 1092–1100. 10.1016/j.chembiol.2017.06.012. [PubMed: 28757181]
11. Rashid OM, & Takabe K (2015). Animal models for exploring the pharmacokinetics of breast cancer therapies. *Expert Opinion on Drug Metabolism & Toxicology*, 11(2), 221–230. 10.1517/17425255.2015.983073. [PubMed: 25416501]
12. Wolf CR, & Henderson CJ (1998). Use of transgenic animals in understanding molecular mechanisms of toxicity. *The Journal of Pharmacy and Pharmacology*, 50(6), 567–574. [PubMed: 9680065]
13. Ho BX, Pek NMQ, & Soh BS (2018). Disease modeling using 3D organoids derived from human induced pluripotent stem cells. *International Journal of Molecular Sciences*, 19(4). 10.3390/ijms19040936.
14. Ozcelikkale A, Moon HR, Linnes M, & Han B (2017). *In vitro* microfluidic models of tumor microenvironment to screen transport of drugs and nanoparticles. *Wiley Interdisciplinary Reviews. Nanomedicine and Nanobiotechnology*, 9(5). 10.1002/wnan.1460.
15. Goel S, England CG, Chen F, & Cai W (2017). Positron emission tomography and nanotechnology: A dynamic duo for cancer theranostics. *Advanced Drug Delivery Reviews*, 113, 157–176. 10.1016/j.addr.2016.08.001. [PubMed: 27521055]

16. Hanahan D, & Weinberg RA (2011). Hallmarks of cancer: the next generation. *Cell*, 144(5), 646–674. 10.1016/j.cell.2011.02.013. [PubMed: 21376230]
17. Dawson MA (2017). The cancer epigenome: concepts, challenges, and therapeutic opportunities. *Science*, 355(6330), 1147–1152. 10.1126/science.aam7304. [PubMed: 28302822]
18. Ricciuti B, De Giglio A, Mecca C, Arcuri C, Marini S, Metro G, et al. (2018). Precision medicine against ALK-positive non-small cell lung cancer: beyond crizotinib. *Medical Oncology*, 35(5), 72. 10.1007/s12032-018-1133-4. [PubMed: 29666949]
19. Hyman DM, Taylor BS, & Baselga J (2017). Implementing genome-driven oncology. *Cell*, 168(4), 584–599. 10.1016/j.cell.2016.12.015. [PubMed: 28187282]
20. Fusco N, & Bosari S (2016). HER2 aberrations and heterogeneity in cancers of the digestive system: implications for pathologists and gastroenterologists. *World Journal of Gastroenterology*, 22(35), 7926–7937. 10.3748/wjg.v22.i35.7926. [PubMed: 27672288]
21. Piulats JM, Guerra E, Gil-Martin M, Roman-Canal B, Gatus S, Sanz-Pamplona R, et al. (2017). Molecular approaches for classifying endometrial carcinoma. *Gynecologic Oncology*, 145(1), 200–207. 10.1016/j.ygyno.2016.12.015. [PubMed: 28040204]
22. Pardoll DM (2012). The blockade of immune checkpoints in cancer immunotherapy. *Nature Reviews. Cancer*, 12(4), 252–264. 10.1038/nrc3239. [PubMed: 22437870]
23. Patel A, & Fong L (2018). Immunotherapy for prostate cancer: where do we go from here?-PART 1: prostate cancer vaccines. *Oncology (Williston Park)*, 32(3), 112–120. [PubMed: 29548065]
24. Kubben N, & Misteli T (2017). Shared molecular and cellular mechanisms of premature aging and aging-associated diseases. *Nature Reviews. Molecular Cell Biology*, 18(10), 595–609. 10.1038/nrm.2017.68. [PubMed: 28792007]
25. Simabuco FM, Morale MG, Pavan ICB, Morelli AP, Silva FR, & Tamura RE (2018). p53 and metabolism: from mechanism to therapeutics. *Oncotarget*, 9(34), 2,378,023,823. 10.18632/oncotarget.25267.
26. Proia DA, & Bates RC (2014). Ganetespib and HSP90: translating preclinical hypotheses into clinical promise. *Cancer Research*, 74(5), 1294–1300. 10.1158/00085472.CAN-13-3263. [PubMed: 24556722]
27. Thakur MK, Heilbrun LK, Sheng S, Stein M, Liu G, Antonarakis ES, et al. (2016). A phase II trial of ganetespib, a heat shock protein 90 Hsp90 inhibitor, in patients with docetaxelpretreated metastatic castrate-resistant prostate cancer (CRPC)-a prostate cancer clinical trials consortium (PCCTC) study. *Investigational New Drugs*, 34(1), 112–118. 10.1007/s10637-015-0307-6. [PubMed: 26581400]
28. Heath EI, Hillman DW, Vaishampayan U, Sheng S, Sarkar F, Harper F, et al. (2008). A phase II trial of 17-allylamino-17-demethoxygeldanamycin in patients with hormone-refractory metastatic prostate cancer. *Clinical Cancer Research*, 14(23), 7940–7946. 10.1158/1078-0432.CCR-08-0221. [PubMed: 19047126]
29. Stratikopoulos EE, & Parsons RE (2016). Molecular pathways: targeting the PI3K Pathway in Cancer-BET Inhibitors to the rescue. *Clinical Cancer Research*, 22(11), 2605–2610. 10.1158/1078-0432.CCR-15-2389. [PubMed: 27250929]
30. Medvetz D, Priolo C, & Henske EP (2015). Therapeutic targeting of cellular metabolism in cells with hyperactive mTORC1: a paradigm shift. *Molecular Cancer Research*, 13(1), 3–8. 10.1158/1541-7786.MCR-14-0343. [PubMed: 25298408]
31. Green DR, Galluzzi L, & Kroemer G (2014). Cell biology. Metabolic control of cell death. *Science*, 345(6203), 1,250,256. 10.1126/science.1250256.
32. Bissell MJ, & Hines WC (2011). Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nature Medicine*, 17(3), 320–329. 10.1038/nm.2328.
33. Bissell MJ, Weaver VM, Lelievre SA, Wang F, Petersen OW, & Schmeichel KL (1999). Tissue structure, nuclear organization, and gene expression in normal and malignant breast. *Cancer Research*, 59(7 Suppl), 1757–1763s discussion 1763s–1764s. [PubMed: 10197593]
34. Bezacny P (2014). Histone deacetylase inhibitors in glioblastoma: pre-clinical and clinical experience. *Medical Oncology*, 31(6), 985. 10.1007/s12032-014-0985-5. [PubMed: 24838514]

35. Fabregat I, Fernando J, Mainez J, & Sancho P (2014). TGF-beta signaling in cancer treatment. *Current Pharmaceutical Design*, 20(17), 2934–2947. [PubMed: 23944366]
36. Moustakas A, Pardali K, Gaal A, & Heldin CH (2002). Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunology Letters*, 82(1–2), 85–91. [PubMed: 12008039]
37. Del Re M, Arrigoni E, Restante G, Passaro A, Rofi E, Crucitta S, et al. (2018). Concise review: resistance to tyrosine kinase inhibitors in non-small Cell lung cancer: the role of cancer stem cells. *Stem Cells*, 36(5), 633–640. 10.1002/stem.2787. [PubMed: 29352734]
38. Melzer C, von der Ohe J, & Hass R (2018). Concise review: crosstalk of mesenchymal stroma/stem-like cells with cancer cells provides therapeutic potential. *Stem Cells*. 10.1002/stem.2829.
39. Pattabiraman DR, & Weinberg RA (2014). Tackling the cancer stem cells - what challenges do they pose. *Nature Reviews. Drug Discovery*, 13(7), 497–512. 10.1038/nrd4253. [PubMed: 24981363]
40. D'Angelo RC, & Wicha MS (2010). Stem cells in normal development and cancer. *Progress in Molecular Biology and Translational Science*, 95, 113–158. 10.1016/B978-0-12-385,071-3.00006-X. [PubMed: 21075331]
41. Ferri KF, & Kroemer G (2001). Organelle-specific initiation of cell death pathways. *Nature Cell Biology*, 3(11), E255–E263. 10.1038/ncb1101-e255. [PubMed: 11715037]
42. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. (2018). Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death and Differentiation*, 25(3), 486–541. 10.1038/s41418-017-0012-4. [PubMed: 29362479]
43. Tsoi J, Robert L, Paraiso K, Galvan C, Sheu KM, Lay J, et al. (2018). Multi-stage differentiation defines melanoma subtypes with differential vulnerability to drug-induced iron-dependent oxidative stress. *Cancer Cell*, 33(5), 890–904 e895. 10.1016/j.ccell.2018.03.017. [PubMed: 29657129]
44. Galluzzi L, Bravo-San Pedro JM, & Kroemer G (2015). Ferroptosis in p53-dependent oncosuppression and organismal homeostasis. *Cell Death and Differentiation*, 22(8), 1237–1238. 10.1038/cdd.2015.54. [PubMed: 26143748]
45. Garg AD, & Agostinis P (2017). Cell death and immunity in cancer: From danger signals to mimicry of pathogen defense responses. *Immunological Reviews*, 280(1), 126–148. 10.1111/immr.12574. [PubMed: 29027218]
46. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, et al. (2012). Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell*, 149(5), 1060–1072. 10.1016/j.cell.2012.03.042. [PubMed: 22632970]
47. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, et al. (2009). Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell*, 138(4), 645–659. 10.1016/j.cell.2009.06.034. [PubMed: 19682730]
48. Mai TT, Hamai A, Hienzsch A, Caneque T, Muller S, Wicinski J, et al. (2017). Salinomycin kills cancer stem cells by sequestering iron in lysosomes. *Nature Chemistry*, 9(10), 1025–1033. 10.1038/nchem.2778.
49. Bernardo MM, Kaplun A, Dzinic SH, Li X, Irish J, Mujagic A, et al. (2015). Maspin expression in prostate tumor cells averts stemness and stratifies drug sensitivity. *Cancer Research*. 10.1158/0008-5472.CAN-15-0234.
50. Ohkubo S, Dalla Via L, Grancara S, Kanamori Y, Garcia-Argaez AN, Canettieri G, et al. (2018). The antioxidant, aged garlic extract, exerts cytotoxic effects on wild-type and multidrug-resistant human cancer cells by altering mitochondrial permeability. *International Journal of Oncology*, 53(3), 1257–1268. 10.3892/ijo.2018.4452. [PubMed: 29956777]
51. Ogita A, Fujita K, & Tanaka T (2012). Enhancing effects on vacuole-targeting fungicidal activity of amphotericin B. *Frontiers in Microbiology*, 3, 100 10.3389/fmicb.2012.00100. [PubMed: 22457662]
52. Feinberg AP (2007). Phenotypic plasticity and the epigenetics of human disease. *Nature*, 447(7143), 433–440. 10.1038/nature05919. [PubMed: 17522677]

53. Holzel M, Bovier A, & Tuting T (2013). Plasticity of tumor and immune cells: a source of heterogeneity and a cause for therapy resistance. *Nature Reviews. Cancer*, 13(5), 365–376. 10.1038/nrc3498. [PubMed: 23535846]
54. Jia D, Jolly MK, Kulkarni P, & Levine H (2017). Phenotypic plasticity and cell fate decisions in cancer: insights from dynamical systems theory. *Cancers (Basel)*, 9(7). 10.3390/cancers9070070.
55. Bernardo MM, Meng Y, Lockett J, Dyson G, Dombkowski A, Kaplun A, et al. (2011). Maspin reprograms the gene expression profile of prostate carcinoma cells for differentiation. *Genes & Cancer*, 2(11), 1009–1022. 10.1177/1947601912440170. [PubMed: 22737267]
56. Zou Z, Anisowicz A, Hendrix MJ, Thor A, Neveu M, Sheng S, et al. (1994). Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. *Science*, 263(5146), 526–529. [PubMed: 8290962]
57. Dzinic SH, Bernardo MM, Li X, Fernandez-Valdivia R, Ho YS, Mi QS, et al. (2017). An essential role of maspin in embryogenesis and tumor suppression. *Cancer Research*, 77(4), 886–896. 10.1158/0008-5472.CAN-16-2219. [PubMed: 27923833]
58. Lockett J, Yin S, Li X, Meng Y, & Sheng S (2006). Tumor suppressive maspin and epithelial homeostasis. [Review]. *Journal of Cellular Biochemistry*, 97(4), 651–660. 10.1002/jcb.20721. [PubMed: 16329135]
59. Bernardo MM, Dzinic SH, Matta MJ, Dean I, Saker L, & Sheng S (2017). The Opportunity of precision medicine for breast cancer with context-sensitive tumor suppressor maspin. *Journal of Cellular Biochemistry*, 118(7), 1639–1647. 10.1002/jcb.25969. [PubMed: 28262971]
60. Kaplun A, Dzinic S, Bernardo M, & Sheng S (2012). Tumor suppressor maspin as a rheostat in HDAC regulation to achieve the fine-tuning of epithelial homeostasis. *Critical Reviews in Eukaryotic Gene Expression*, 22(3), 249–258. [PubMed: 23140166]
61. Li X, Yin S, Meng Y, Sakr W, & Sheng S (2006). Endogenous inhibition of histone deacetylase 1 by tumor-suppressive maspin. *Cancer Research*, 66(18), 9323–9329. 10.1158/0008-5472.CAN-06-1578. [PubMed: 16982778]
62. Zhang M, Hendrix MJC, Pemberton PA, Sakr WA, & Sheng S (2017). An Essential Role of Maspin in Embryogenesis and Tumor Suppression-Response. *Cancer Research*, 77(18), 5208–5210. 10.1158/0008-5472.CAN-17-1254. [PubMed: 28877938]
63. Biliran H Jr., & Sheng S (2001). Pleiotrophic inhibition of pericellular urokinase-type plasminogen activator system by endogenous tumor suppressive maspin. *Cancer Research*, 61(24), 8676–8682. [PubMed: 11751384]
64. Jiang N, Meng YH, Zhang SL, Mensah-Osman E, & Sheng SJ (2002). Maspin sensitizes breast carcinoma cells to induced apoptosis. *Oncogene*, 21(26), 4089–4098. 10.1038/sj.onc.1205507. [PubMed: 12037665]
65. Cher ML, Biliran HR, Bhagat S, Meng YH, Che MX, Lockett J, et al. (2003). Maspin expression inhibits osteolysis, tumor growth, and angiogenesis in a model of prostate cancer bone metastasis. *Proceedings of the National Academy of Sciences of the United States of America*, 100(13), 7847–7852. 10.1073/pnas.1331360100. [PubMed: 12788977]
66. Domingo-Domenech J, Vidal SJ, Rodriguez-Bravo V, Castillo-Martin M, Quinn SA, Rodriguez-Barrueco R, et al. (2012). Suppression of acquired docetaxel resistance in prostate cancer through depletion of notch- and hedgehog-dependent tumor-initiating cells. *Cancer Cell*, 22(3), 373–388. 10.1016/j.ccr.2012.07.016. [PubMed: 22975379]
67. Hwang C (2012). Overcoming docetaxel resistance in prostate cancer: a perspective review. *Therapeutic Advances in Medical Oncology*, 4(6), 329–340. 10.1177/1758834012449685. [PubMed: 23118808]
68. O'Neill AJ, Prencipe M, Dowling C, Fan Y, Mulrane L, Gallagher WM, et al. (2011). Characterization and manipulation of docetaxel resistant prostate cancer cell lines. *Molecular Cancer*, 10, 126 10.1186/1476-4598-10-126. [PubMed: 21982118]
69. Li XH, Chen D, Yin SP, Meng YH, Yang HJ, Landis-Piowar KR, et al. (2007). Maspin augments proteasome inhibitor-induced apoptosis in prostate cancer cells. *Journal of Cellular Physiology*, 212(2), 298–306. 10.1002/Jcp.21102. [PubMed: 17458898]

70. Tahmatzopoulos A, Sheng S, & Kyprianou N (2005). Maspin sensitizes prostate cancer cells to doxazosin-induced apoptosis. *Oncogene*, 24(34), 5375–5383. 10.1038/sj.onc.1208684. [PubMed: 16007219]
71. Zhang Y, Liu L, Li F, Wu T, Jiang H, Jiang X, et al. (2017). Salinomycin Exerts Anticancer Effects on PC-3 Cells and PC-3-Derived Cancer Stem Cells *In vitro* and *In vivo*. *BioMed Research International*, 4, 101–653. 10.1155/2017/4101653.

Fig. 1. Multifaceted heterogeneity in cancer phenotypes as the cause of functional gains, survival vulnerabilities, and drug resistance. **a** Key cellular operatives and mechanisms underlying the functional gains and survival vulnerabilities of cancer cells. **b** The heterogeneity of cancer cells in the continuum of tumor progression and metastasis that underlie the insufficiency of drugs that targeted subpopulations, but not the whole population of cancer cells. ★ Marks cancer stem cells

Fig. 2. Scheme 2D/3D/mammosphere recapitulates distinct functional states of cancer cells for robust and comprehensive drug screening. **a** The effects of maspin on context-dependent prostate tumor cell phenotypic plasticity. **b** The drug response of phenotypically distinct subpopulations of prostate cancer cells in the presence or absence of maspin