Minireview

Modeling adaptive drug resistance of colorectal cancer and therapeutic interventions with tumor spheroids

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Impact statement

Chemoresistance is a major challenge against sustained and effective treatments of cancer patients with primary or metastatic disease. Modern cancer treatments designed based on mechanisms of drug resistance increase treatment benefits and improve outcomes for patients. A technological challenge for the design and testing of specific treatment strategies is the need for physiologic tumor models that reproduce the biology of native tumors. *In vitro* tumor models are also expected to aid clinicians with selecting specific drugs from an increasingly larger pool of cancer drugs with similar or complementary mechanisms of action. We present the utility of a 3D tumor spheroid model to study drug resistance in colorectal cancer phenotypically and mechanistically in a clinically relevant cyclic treatment regimen that mimics how patients receive chemotherapy. We demonstrate the feasibility of a design-driven approach to develop specific drug combinations that offer long-term benefits.

Abstract

Drug resistance is a major barrier against successful treatments of cancer patients. Various intrinsic mechanisms and adaptive responses of tumor cells to cancer drugs often lead to failure of treatments and tumor relapse. Understanding mechanisms of cancer drug resistance is critical to develop effective treatments with sustained anti-tumor effects. Three-dimensional cultures of cancer cells known as spheroids present a biologically relevant model of avascular tumors and have been increasingly incorporated in tumor biology and cancer drug discovery studies. In this review, we discuss several recent studies from our group that utilized colorectal tumor spheroids to investigate responses of cancer cells to cytotoxic and molecularly targeted drugs and uncover mechanisms of drug resistance. We highlight our findings from both short-term, one-time treatments and long-term, cyclic treatments of tumor spheroids and discuss mechanisms of adaptation of cancer cells to the treatments. Guided by mechanisms of resistance, we demonstrate the feasibility of designing specific drug combinations to effectively block growth and resistance of cancer cells in spheroid cultures. Finally, we conclude with our perspectives on the utility of three-dimensional tumor models and their shortcomings and advantages for phenotypic and mechanistic studies of cancer drug resistance.

Keywords: Drug resistance, 3D tumor model, colorectal cancer, cyclic treatment, compensatory signaling, combination treatments

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Introduction

Cancer drug resistance

Resistance to cytotoxic chemotherapy and molecularly targeted drugs commonly occurs in a majority of cancer patients and significantly detracts from the efficacy of the treatments.[1] Drug resistance is broadly classified as intrinsic or acquired.[2] Intrinsic resistance is mediated by pre-existing heterogeneity of cancer cells in the bulk tumor. Tumors inherently contain heterogeneous populations of cancer cells with genetic and functional differences. While cytotoxic chemotherapeutics primarily target actively proliferating cells, the use of modern targeted therapies following analysis of tumor biopsies aims to target main molecular driver(s) of tumorigenesis. Therefore, non-proliferative cells in tumors, slow-cycling cells due to nutrients limitations, quiescent stem-like cells, and cells not represented as the main driver(s) of tumor growth in a biopsy analysis often escape the therapies and promote tumor relapse.[3] Acquired drug resistance, on the other hand, arises during the course of treatments and exposure of cancer cells to drugs. Mechanisms of acquired resistance are diverse and include mutations,[4] alterations,[5,6] or over-activation of the therapeutic target,[7] feedback activation of

compensatory oncogenic signaling pathways,[8] epigenetically regulated drug tolerance,[9] overexpression of efflux transporter pumps,[10] and hypoxia in the tumor microenvironment. [11,12]

The advent of powerful techniques for molecular profiling of tumors and stratification of patients, along with enhanced capabilities for high throughput screening of cancer drugs in preclinical models, have led to unprecedented opportunities to understand these resistance mechanisms and develop treatment strategies, such as rational drug combinations, to overcome resistance. Additionally, in the past few decades, there have been major investments in oncology drug discovery that resulted in a greater number of drugs available to clinicians. These efforts have led to improvements in outcomes for cancer patients, and according to the American Cancer Society, the death rate from cancer has declined by 29% from 1991 to 2017 in the US.[13] Nevertheless, cancer remains the second leading cause of death in the US, emphasizing the need for continuous investments to enable new discoveries that lead to more effective treatments.

Models of cancer drug resistance

Preclinical studies of efficacy and safety of anti-cancer compounds and their mechanisms of action are routinely performed in cell culture and animal models. Monolayer (2D) cell cultures provides a convenient approach to screen large arrays of chemical compounds against cancer cells and to develop resistant lines of cells, for example, by continuous, long-term, low-dose drug exposure of cells. A report in 2010 estimated that more than 80% of cancer biologists in academic and industrial settings relied on 2D monocultures and co-cultures of cells prior to animal studies.[14] However, the lack of complex tissue architecture and close cell–cell contacts and cell-extracellular matrix (ECM) interactions in these cell culture models is considered a major roadblock against identifying cancer drug candidates with successful outcomes in clinical trials.[15] On the other hand, animal models present a fully physiologic system to study bioavailability, therapeutic efficacy, and dose-limiting toxicity of cancer drugs.[16] In recent years, patient-derived tumor xenografts (PDX) have also been used to maintain and passage primary tumor cells in immunocompromised mice without exposing the cells to *in vitro* cultures. PDX models largely maintain genetic identity and cellular heterogeneity of their parental tumors and may also reproduce their clinical drug responses.[17–20] However, low-to-moderate success rates of developing tumor xenografts that often takes several months, significant differences in the tumor environments between the stroma of native and xenograft tumors, difficulty of conducting mechanistic studies with animals, and incompatibility of animal models with screening of drug compounds limit their utility for mechanistic studies of drug resistance and identifying treatments to overcome it. [21] Therefore, there has been a growing interest in the research community and the pharmaceutical industry to transition from the overly simplistic 2D cell cultures to models that more closely recapitulate the architecture and biology of native tumors, while avoiding the disadvantages of animal models.[22] To address this need, several 3D cell culture models have been developed to enable mechanistic studies of drug response and resistance of cancer cells, allow testing of arrays of drugs and their combinations to identify treatments that block drug resistance, and improve the efficacy and durability of cancer therapies.

Scope of this review

In the past decade, there has been an increasing interest in academia, national research laboratories, NCI-designated comprehensive cancer centers, and major pharmaceutical companies to develop or use 3D tumor models for mechanistic studies of tumor biology, design and test new treatment modalities, and develop targeted therapies that are tailored to specific cohorts of cancer patients. Various 3D tumor models have been developed that can generally be classified as follows. (i) Tumor spheroids are 3D compact aggregates of cancer cells that form spontaneously due to cell–cell adhesion in the absence of a cell-adherent surface, or within natural or synthetic matrices. Tumor spheroids are often made using cancer cell lines alone or as an intermixed co-culture of cancer and stromal cells. There are several techniques to develop free-floating spheroids, including spinner flask, aqueous-two-phase system, hanging drop, and magnetic levitation.[23] Straightforward initiation and maintenance of spheroids is a major advantage for mechanistic and drug screening studies. However, genetic shifts of cancer cell lines from their parental tumor cells make the use of spheroids of cancer cell lines less attractive for translational studies, although spheroids remain a major tool for basic cancer research studies. (ii) Organoids are 3D cultures that are developed from self-assembly of pluripotent or adult stem cells under organogenesis cues in Matrigel, or directly using patient-derived materials.[24,25] Organoids mimic the architecture and cellular composition and organization of the respective tissues. More importantly and from a disease modeling point of view, organoids that develop from patient tumor biopsies or resections recapitulate the histological and functional properties of the parental tumors,[26,27] making them a valuable tool to understand disease mechanisms and develop therapies for individual cancer patients.[28] Relatively, long time needed for the formation of tumor organoids and the difficulty to mass produce them remain major drawbacks. (iii) Microfluidics technology has been useful to develop compartmentalized 3D tumor models. Often made using polydimethylsiloxane (PDMS) soft lithography, microfluidic devices enable culturing cancer cells and stromal cells in separate microchannels or microchambers to model processes such as chemotactic invasion of cancer cells and angiogenesis, and to perform drug testing studies.[29,30] The need for high expertise of users to develop and maintain microfluidic cell cultures, difficulty to access cells for downstream analytical studies, and incompatibility with standard robotic pipetting tools and instruments for biochemical analysis have limited the use of microfluidic tumor models primarily as a laboratory research tool.

Over the past several years, our group has developed tumor spheroid models of breast and colorectal cancers for mechanistic studies of drug resistance and to test and identify effective treatment strategies. This review highlights our recent studies that demonstrate the utility of tumor spheroids to model adaptive resistance of colorectal cancer (CRC) cells to targeted therapies and develop design-driven approaches to block drug resistance. Our focus on CRC is because it is the third most common cancer and the third leading cause of cancer mortality in the United States.[31] Similar to other cancers, genetic and epigenetic factors cause CRC tumorigenesis.[32] According to Cancer Genome Atlas Network in 2012 and an international consortium in 2015,[33] approximately 50% of CRCs contain frequent gain-of-function mutations in RAS and RAF and 25% have mutations in PI3K/Akt pathway.[34,35] These mutations lead to hyper-activity of highly conserved signaling pathways including RAF/MEK/ERK, PI3K/Akt, and JAK/Stat, resulting in aberrant proliferation and survival of CRC cells. The use of molecular inhibitors of these pathways in single-agent therapies has been sought to suppress CRC tumorigenesis. Despite initial anti-tumor effects of the inhibitors,[36] tumor cells often develop resistance to MEK and RAF inhibitors (MEKi and RAFi) through mechanisms such as feedback activation of compensatory signaling pathways or upstream receptor tyrosine kinases (RTKs),[37,38] further mutations and amplification of the target gene,[39,40] and gain of a stem cell-like state.[41] Below, we highlight the utility of tumor spheroids as a preclinical tool to model such complex molecular events and examine the effectiveness of treatment strategies against therapy resistance of CRC cells.

Quantitative analysis of high-throughput drug screening with tumor spheroids

Polymeric aqueous two-phase systems (ATPS) have been demonstrated as a versatile tool for cell and protein micropatterning.[42–45] Atefi *et al.*[46,47] developed a tumor spheroid microtechnology using an ATPS with 5.0% (w/v) polyethylene glycol (PEG) and 6.4% (w/v) dextran (DEX) polymers. A nanodrop of the denser aqueous DEX phase containing cancer cells was dispensed into the immersion aqueous PEG phase. It was demonstrated that an ultralow interfacial tension of ~30 μJ/m^2 between the two aqueous phases was essential to retain cells within the nanodrop phase to spontaneously self-assemble into a spheroid.[48,49] Adapting this technology to robotic liquid handling allowed convenient generation of consistently sized tumor spheroids in standard microwell plates.[50] To establish the feasibility of this technology for drug screening applications, Thakuri *et al.*[51] conducted a dose-dependent screening of 25 different anticancer compounds against HT-29 CRC spheroids (Figure 1(a)), and optimized a standard Prestoblue assay to quantify cellular responses, as shown for selumetinib (MEKi) in Figure 1(b). From each dose–response curve, half-maximum inhibitory concentration (IC_{50}) and maximum inhibition (E_{max}) values, which respectively are classical measures of potency and efficacy of a drug, were computed. A low IC_{50} value indicated that the drug was effective at low concentrations, whereas E_{max}

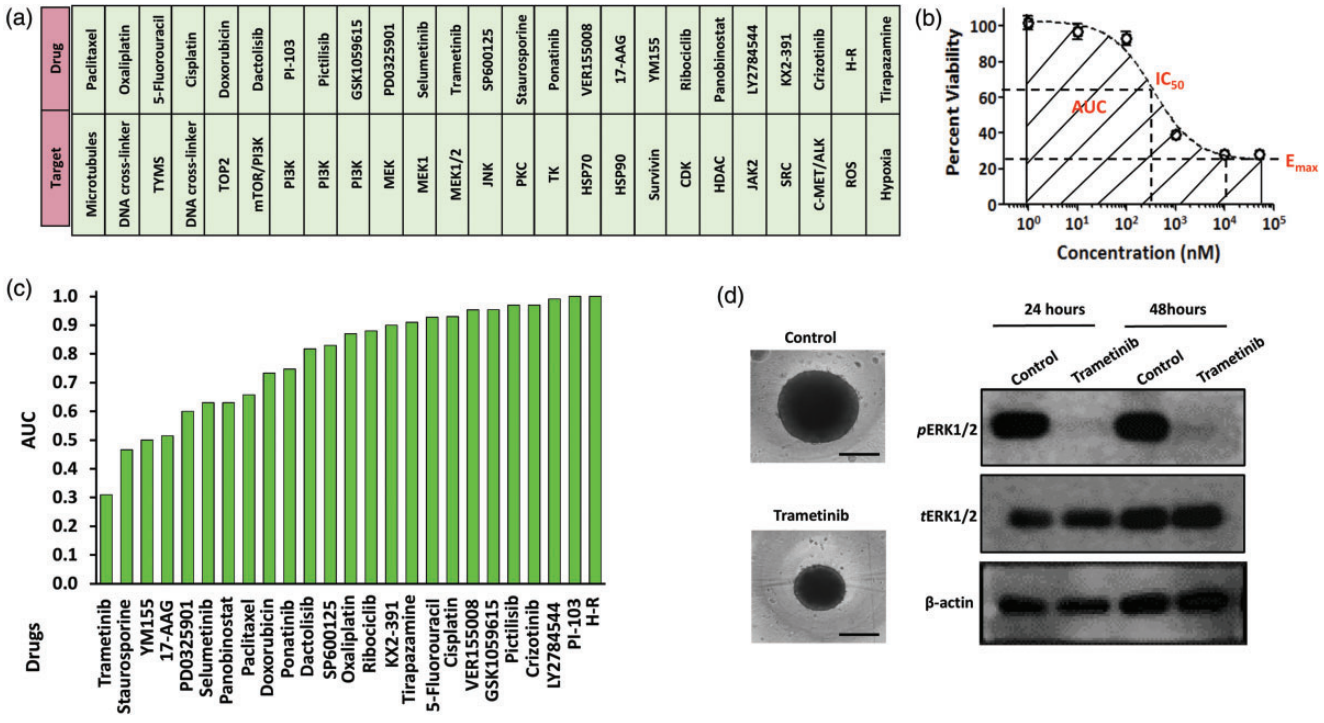

Figure 1. (a) List of 25 drug compounds used to screen against colorectal tumor spheroids. Molecular targets of the compounds are also shown. (b) Dose–response of HT-29 tumor spheroids to selumetinib treatment. Half-maximum inhibitory concentration (IC_{50}), maximum inhibition (E_{max}), and area under the curve (AUC) metrics used for multiparametric analysis of treatment response are shown. (c) Ranking of effectiveness of 25 compounds against HT-29 spheroids based on the AUC score. (d) Trametinib treatment at a 100 nM concentration significantly reduced the size of HT-29 spheroids and completely blocked ERK1/2 signaling in HT-29 spheroids. Scale bar is 250 μm. (A color version of this figure is available in the online journal.)

varied between 1 and 0 with smaller values indicating greater cell death. This screening identified a MEK1/2 inhibitor, trametinib, as the most potent and effective compound from this set of drugs, evident from an IC_{50} value of 0.0015 μM and an E_{max} value of 0.21. To combine drug potency and efficacy into a single parameter and quantitatively compare different drugs, an area under the dose–response curve (AUC) was computed for each compound against the spheroids. AUC values were normalized to a 0–1 range with values approaching zero indicating both high potency and efficacy. Consistent with the gain-of function BRAF mutation and high activity of the RAF/MEK/ERK signaling pathway in HT-29 CRC cells, trametinib gave the lowest AUC of 0.31 and ranked first among the 25 compounds used against HT-29 spheroids (Figure 1(c)). A single-dose of trametinib completely blocked ERK1/2 phosphorylation and effectively inhibited growth of HT-29 spheroids (Figure 1(d)). Other inhibitors of MAPK pathways, i.e., PD0325901 and selumetinib, resulted in AUC scores of 0.59 and 0.63, respectively. Thus, trametinib was the most effective MAPK inhibitor against the CRC spheroids in a single-agent, one-time treatment regimen.

Although biochemical assays are widely used to determine drug responses of cancer cells, the assays are often terminal, and the samples can no longer be used. This approach works well with short-term, one-time drug treatment experiments. However, to study long-term effectiveness of drugs on cancer cells and evaluate changes in drug responsiveness of cells over time, different samples should be prepared for different time points. When screening large sets or libraries of drugs, this introduces operational challenges and necessitates creating thousands to tens of thousands of samples to enable dose-dependent screening of compounds with enough replicates to facilitate statistical analysis. To address this issue, Thakuri *et al.*[52] showed that analysis of phase-contrast images of spheroids can be used to reliably predict the growth of the spheroids and their responses to targeted therapies. Using four different inhibitors of protein kinases (trametinib, sorafenib, ponatinib, dactolosib), it was shown that dose-dependent reduction in the size of HT-29 spheroids from morphological analysis of spheroids strongly correlates with the results from drug response analysis of cells using a biochemical assay (Figure 2). It is important to note that the morphological analysis of drug responses of spheroids was only valid when treatments resulted in shrinking of spheroids and did not disintegrate them. This approach is often useful when using molecular inhibitors at low-to-moderate concentrations.

Modeling adaptive drug resistance of CRC cells to targeted therapies

Using a pulsed-dosing regimen to mimic intermittent cycles of chemotherapy administered to patients,[53,54] Thakuri *et al.*[55] treated CRC spheroids with inhibitors of MAPK pathways (RAFi AZ628, MEKi trametinib, ERKi SCH772984) cyclically with recovery intervals in between (Figure 3(a)). Responses of HCT116 CRC spheroids to long-term, cyclic treatments with the LD_{50} concentrations of the inhibitors were evaluated using morphological analysis. The inhibitors potently blocked proliferation of cancer cells and reduced the size of spheroids during the first treatment round (T1) (Figure 3(b)). However, the inhibitors became significantly less effective during the subsequent rounds (T2, T3, T4) and despite repeated treatments, the HCT116 spheroids grew larger. A similar result was obtained with HT-29 CRC spheroids under cyclic treatments with different MEKi,[56] confirming that CRC cells adapt to targeted therapies. Due to extensive crosstalk between MAPK and PI3K/Akt pathways in different cancers,[57] the activity of PI3K/Akt pathway in CRC cells treated with the MAPKi was examined (Figure 3(c)). All three inhibitors significantly reduced pERK1/2 levels, with trametinib being the most effective and downregulating ERK1/2 activity in HCT116 spheroids by ~85%. Nevertheless, the treatments led to significant increases in the pAkt levels (Figure 3(d) and (e)). Experiments with HT-29 spheroids also led to a similar outcome.[56] Thus, the use of tumor spheroids with a clinically relevant cyclic treatment regimen established that the MAPK pathway inhibitors activate compensatory PI3K/Akt signaling in $BRAF^{mut}$ (HT-29) and $KRAS^{mut}$ (HCT116) CRCs, rendering treatments ineffective. These results were also consistent with a prior study that treated a large panel of $KRAS^{mut}$ CRC cell lines with a MEKi, resulting in PI3K/Akt pathway activity due to either activating mutations in PIK3CA or loss-of-function mutation in PTEN.[58]

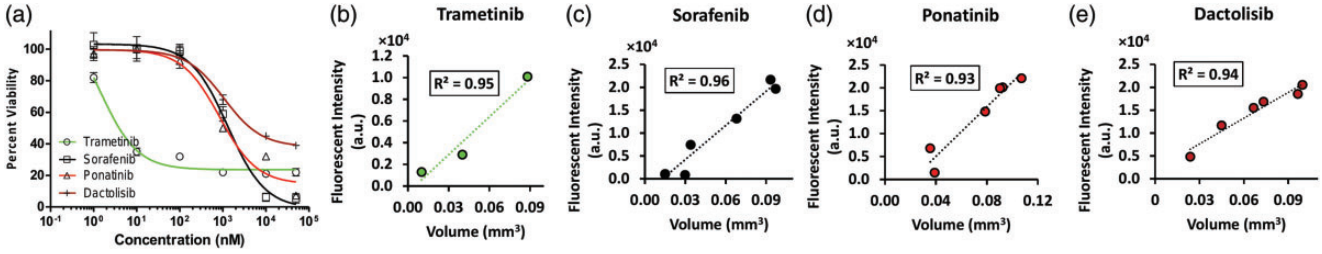

Figure 2. (a) Dose–response of HT-29 spheroids to four different protein kinase inhibitors. (b–e) Correlation between the average values of fluorescent signal from Prestoblue metabolic activity assay and volume of spheroids from morphological images ($n = 14$). R^2 represents goodness-of-the-fit parameter. Different data points in each graph represent different drug concentrations. Only those concentrations that did not disintegrate the spheroids were considered. (A color version of this figure is available in the online journal.)

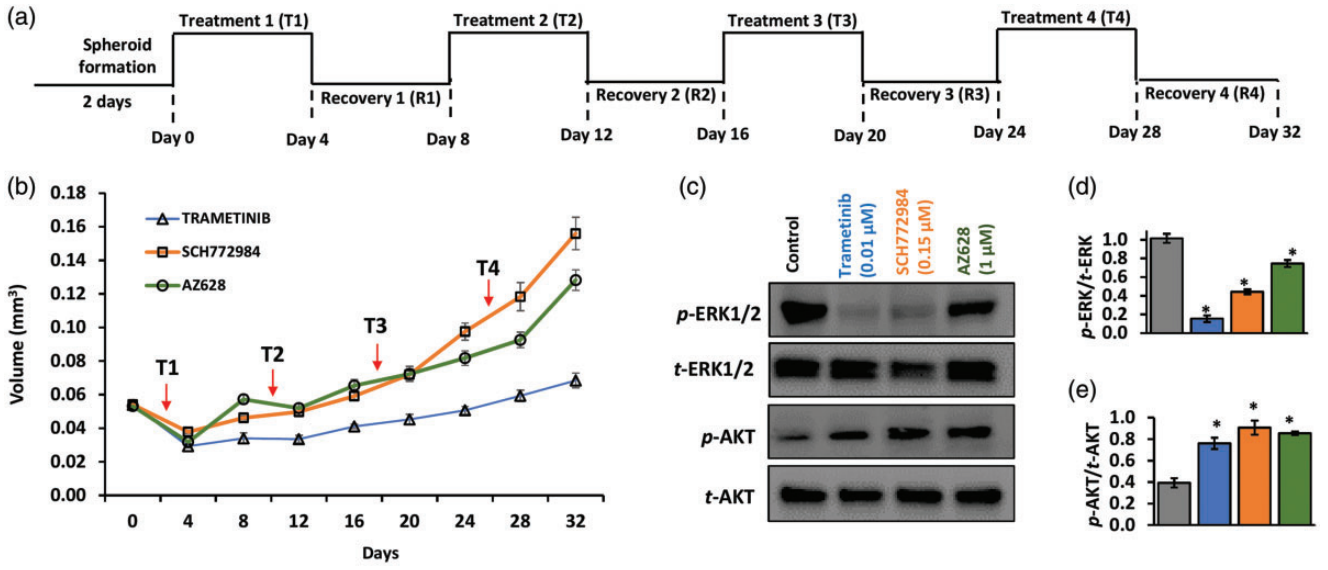

Figure 3. (a) Cyclic treatment and recovery regimen used to model adaptive drug resistance of cancer cells. (b) HCT116 spheroids were cyclically treated with inhibitors of MEK, ERK, and RAF and their volumes were measured at nine different time points over the 32-day treatment period. Kinetics of growth of spheroids shows that despite significant effects of the treatments during T1, cells quickly adapted and developed resistance, as evident from increase in the size of tumor spheroids. (c) Western blots for ERK1/2 and Akt activities of HCT116 spheroids show that although the treatments downregulate ERK activity, cells activate Akt signaling to bypass the treatments. (d–e) Quantified results of p-ERK/t-ERK and p-AKT/t-AKT in the vehicle control and treated spheroids. (A color version of this figure is available in the online journal.)

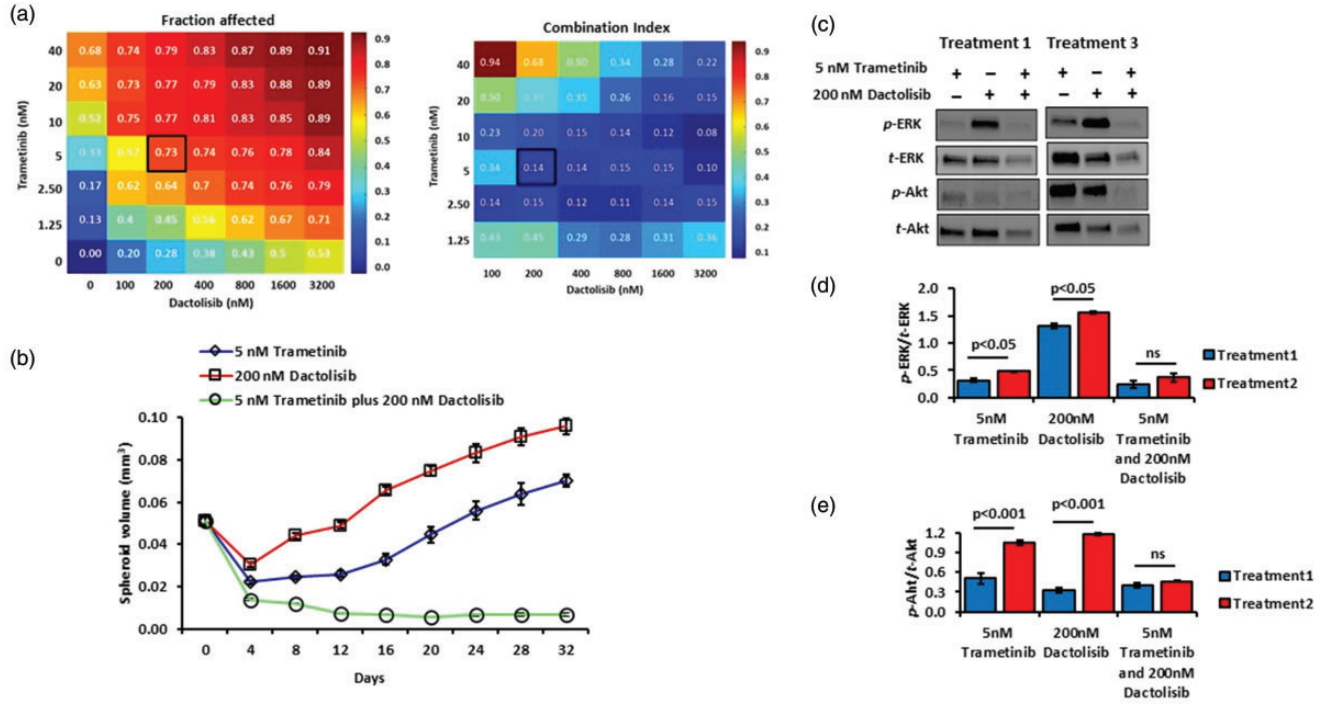

Figure 4. (a) Heatmaps of fraction affected and combination index from combination treatment of HCT116 spheroids with trametinib and dactolisib. (b) The combination treatment effectively suppressed growth of HCT116 spheroids in long-term cyclical regimen, whereas individual treatments with trametinib or dactolisib were ineffective. (c–e) Representative Western blots of activities of ERK1/2 and AKT in HCT116 spheroids under single-agent and combination treatments from T1 and T3 rounds, and quantified results from the blots. (A color version of this figure is available in the online journal.)

Design-driven combination treatments to block adaptive drug resistance

Guided by the molecular mechanism of resistance of CRC spheroids to the MAPKi, Thakuri *et al.*[55,56] examined the effectiveness of simultaneous blocking of both MAPK and PI3K/Akt pathways. Following a dose-dependent screening of several inhibitors of PI3K/Akt/mTOR pathway against the HT-29 and HCT116 cells, dactolisib was selected as the most potent inhibitor for combination treatments

with three different MEKi (trametinib, selumetinib, and PD0325091). First, a synergy analysis following short-term, dose-dependent combination treatments was used to determine synergistic combinations of each pair of inhibitors (Figure 4(a)). A combination index (CI) was used to define synergy when $0<CI<1$, with $CI\rightarrow 0$ indicating a strong synergy. Based on this metric, a highly synergistic concentration pair of each MEKi and dactolisib was selected for long-term, cyclic treatments of CRC spheroids.[56] For example with HCT116 spheroids, a 5 nM trametinib and 200 nM dactolisib pair that gave a CI values of 0.14 was selected (boxed cells in the heatmaps of Figure 4(a)). With both HT-29 and HCT116 cells, the combination treatments very effectively suppressed the growth of tumor spheroids during four weeks of culture (Figure 4(b)). Molecular analysis validated that the combination treatment blocked adaptive resistance of cancer cells to the MAPKi and simultaneously inhibited signaling of both pathways in CRC cells in the long-term cyclic treatments (Figure 4(c) and (e)). Overall, these studies demonstrated the feasibility of identifying mechanisms of adaptive resistance of CRC cells in tumor spheroids to single-agent targeted therapies and designing combination treatments that block crosstalk among oncogenic pathways and tumor growth.

Perspectives

Despite a significant increase in the number of available cancer drugs and development of new treatment strategies, drug resistance remains a major cause of failure of chemotherapies and targeted therapies.[2] Understanding resistance mechanisms in preclinical studies is critical to design more effective therapies. We reviewed several recent studies from our group on adaptive drug resistance of CRC cells. We had previously established that spheroid cultures of cancer cells reproduce various morphological and biological characteristics of solid tumors and present a simple, yet powerful tool for mechanistic cancer research.[59] Our recent studies capitalized on this technology and demonstrated the utility of tumor spheroids to identify specific mechanisms of drug resistance that render single-agent targeted therapies ineffective. Uniquely, we recreated adaptation of cancer cells to treatments using a cyclic treatment/recovery regimen. Molecular inhibitors (e.g. a clinically used MEK inhibitor, trametinib) that had a strong anti-proliferative effect against CRC cells in short-term treatments became progressively less effective during cyclic treatments due to activation of a compensatory feedback signaling. Guided by the molecular mechanism of resistance of CRC cells to single-agent therapies with MAPK pathway inhibitors, we designed several combination treatments to target both the initially active MAPK pathway and feedback-activated PI3K/Akt pathway. The use of tumor spheroids in 384-microwell plates enabled high throughput, dose-dependent screening to identify highly synergistic pairs of drugs that effectively blocked signaling through both pathways in long-term cyclic treatments of CRC spheroids. Overall, our findings highlight the importance of incorporating clinically relevant regimens in mechanistic preclinical studies of drug resistance and to test and identify synergistic drug combinations against tumor growth.

While we demonstrated that compensatory kinase signaling accounts for adaptive resistance of colorectal cancer spheroids to MEKi, our studies did not consider potential effects of hypoxia on therapy resistance. We previously showed that colorectal tumor spheroids of $\sim$400 µm diameter have a non-uniform distribution of Ki-67^{+} proliferative cells toward the periphery of the spheroids,[60] implying oxygen and nutrients deficiency and potentially hypoxia in the core region. During the cyclic treatment and recovery regimen, spheroids grew even larger. Therefore, it remains an open question to investigate to what extent hypoxia may contribute to drug resistance of CRC spheroids relative to the feedback signaling of kinase pathways. We note that the feedback signaling activation only occurred in spheroids cyclically treated with MEKi. The vehicle control, untreated spheroids that were significantly larger than the treated spheroids and more likely to have a hypoxic core did not develop compensatory kinase signaling or had a significantly lower activation of PI3K/Akt pathway compared to the treated spheroids. Therefore, this suggests that resistance to cyclic treatments with MEKi was due to feedback activation of PI3K/Akt pathway and not driven by hypoxia in spheroids.

Our studies primarily focused on effects of drugs, used alone or in combinations, on proliferative activities of cancer cells in spheroid cultures. Although blocking cancer cell proliferation is critical to inhibit tumor growth and relapse, other pro-metastatic processes such as matrix invasion of cancer cells, which leads to local invasion and eventually distant metastasis, should also be considered in the design of treatment regimens. In a recent study, we demonstrated that simultaneous inhibition of MAPK and PI3K/Akt pathways using MEK and EGFR inhibitors blocks growth of CRC spheroids but not invasion of cancer cells when the treated spheroids were embedded in collagen.[61] We showed that the invasive phenotype was driven by activation of STAT signaling and that sequential treatment of spheroids with a STAT inhibitor was necessary to also block cancer cell invasiveness. This study highlighted the complexity of events in the tumor microenvironment and the importance of examining how specific drugs and their combinations affect different pro-metastatic processes. Although animal models allow capturing a variety of events such as tumor growth, migration and invasion, angiogenesis, immune cell infiltration of tumors, as well as metastatic dissemination of cancer cells, significant challenges of conducting mechanistic studies with animal models and their incompatibility with screening of drugs and their combinations underscore a major role for 3D cell-based assays in preclinical studies.

It is now well-recognized that the tumor stroma plays major roles in cancer progression.[62] The stroma in solid tumors is a complex environment that contains various types of cells such as fibroblasts, endothelial cells, immune cells, and different non-cellular components including the extracellular matrix proteins, growth factors, cytokines, and chemokines. Physical and biochemical interactions of cancer cells with the tumor stroma promote

pro-metastatic functions of cancer cells and resistance to therapeutics.[63–65] As such, future studies should expand on the spheroid model using other components of the tumor microenvironment and examine effects of specific interactions, e.g. fibroblasts—cancer cells or immune cells—cancer cells, on drug resistance and other processes, as we recently demonstrated using an organotypic tumor model.[45] Developing such complex tumor models and incorporating them in our cyclic treatment regimen will enable mechanistic studies of tumor-stromal interactions that shape the progression of events in the tumor microenvironment and lead to therapy resistance and tumor relapse. This model can also help examine the efficacy of various drug combinations, such as molecular inhibitors with or without cytotoxic chemotherapeutics, against cancer cells and the tumor microenvironment, an approach that is being pursued in different clinical trials.[66,67]

Considering that treating cancers with a single drug almost always fails due to drug resistance, combination therapies are crucial to improve outcomes for patients.[68] Consistent with clinical trials in different cancers,[69,70] our preclinical studies with the CRC tumor spheroid model demonstrated significant anti-tumor benefits of combination therapies. Nevertheless, a major barrier against giving two or more drugs simultaneously to patients is increased off-target toxicity to normal cells and tissues. Excessive toxicity of drug combinations has led to failure of several clinical trials.[71–73] Therefore, a major consideration in preclinical studies should be given to potential toxic effects of drugs on different organs. This has been mainly studied preclinically in animal studies by monitoring the animal weight during treatments and histological examination of different organs after the termination of treatments. However, advances in 3D cellular models including the organs-on-a-chip technology will enable evaluating treatment-induced toxicity more conveniently and dynamically to identify effective drug combinations with reduced toxic effects for clinical trials.

AUTHORS' CONTRIBUTIONS

All authors participated in the design and writing of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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REFERENCES

1. Vasan N, Baselga J, Hyman DM. A view on drug resistance in cancer. *Nature* 2019;**575**:299–309
2. Groenendijk FH, Bernards R. Drug resistance to targeted therapies: Déjà vu all over again. *Mol Oncol* 2014;**8**:1067–83
3. Housman G, Byler S, Heerboth S, Lapinska K, Longacre M, Snyder N, Sarkar S. Drug resistance in cancer: an overview. *Cancers* 2014;**6**:1769–92
4. Lee JJ, Sholl LM, Lindeman NI, Granter SR, Laga AC, Shivdasani P, Chin G, Luke JJ, Ott PA, Hodi FS, Mihm MC, Lin JY, Werchniak AE, Haynes HA, Bailey N, Liu R, Murphy GF, Lian CG. Targeted next-generation sequencing reveals high frequency of mutations in epigenetic regulators across treatment-naïve patient melanomas. *Clin Epigenetics* 2015;**7**:59
5. Ellis LM, Hicklin DJ. Resistance to targeted therapies: refining anticancer therapy in the era of molecular oncology. *Clin Cancer Res* 2009;**15**:7471–8
6. Aldonza MBD, Ku J, Hong JY, Kim D, Yu SJ, Lee MS, Prayogo MC, Tan S, Kim D, Han J, Lee SK, Im SG, Ryu HS, Kim Y. Prior acquired resistance to paclitaxel relays diverse EGFR-targeted therapy persistence mechanisms. *Sci Adv* 2020;**6**:eaav7416
7. Neel DS, Bivona TG. Resistance is futile: overcoming resistance to targeted therapies in lung adenocarcinoma. *NPJ Precis Oncol* 2017;**1**:3
8. Floros KV, Hata AN, Faber AC. Investigating new mechanisms of acquired resistance to targeted therapies: if you hit them harder, do they get up differently? *Cancer Res* 2020;**80**:25–6
9. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, McDermott U, Azizian N, Zou L, Fischbach MA, Wong K-K, Brandstetter K, Wittner B, Ramaswamy S, Classon M, Settleman J. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* 2010;**141**:69–80
10. Ng KP, Hillmer AM, Chuah CTH, Juan WC, Ko TK, Teo ASM, Ariyaratne PN, Takahashi N, Sawada K, Fei Y, Soh S, Lee WH, Huang JWJ, Allen JCJ, Woo XY, Nagarajan N, Kumar V, Thalamuthu A, Poh WT, Ang AL, Mya HT, How GF, Yang LY, Koh LP, Chowbay B, Chang C-T, Nadarajan VS, Chng WJ, Than H, Lim LC, Goh YT, Zhang S, Poh D, Tan P, Seet J-E, Ang M-K, Chau N-M, Ng Q-S, Tan DSW, Soda M, Isobe K, Nöthen MM, Wong TY, Shahab A, Ruan X, Cacheux-Rataboul V, Sung W-K, Tan EH, Yatabe Y, Mano H, Soo RA, Chin TM, Lim W-T, Ruan Y, Ong ST. A common BIM deletion polymorphism mediates intrinsic resistance and inferior responses to tyrosine kinase inhibitors in cancer. *Nat Med* 2012;**18**:521–8
11. Jing X, Yang F, Shao C, Wei K, Xie M, Shen H, Shu Y. Role of hypoxia in cancer therapy by regulating the tumor microenvironment. *Mol Cancer* 2019;**18**:1–15
12. Xu K, Zhan Y, Yuan Z, Qiu Y, Wang H, Fan G, Wang J, Li W, Cao Y, Shen X, Zhang J, Liang X, Yin P. Hypoxia induces drug resistance in colorectal cancer through the HIF-1α/miR-338-5p/IL-6 feedback loop. *Mol Ther* 2019;**27**:1810–24
13. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin* 2017;**67**:7–30
14. Hutmacher DW. Biomaterials offer cancer research the third dimension. *Nat Mater* 2010;**9**:90–3
15. Waring MJ, Arrowsmith J, Leach AR, Leeson PD, Mandrell S, Owen RM, Pairaudeau G, Pennie WD, Pickett SD, Wang J, Wallace O, Weir A. An analysis of the attrition of drug candidates from four major pharmaceutical companies. *Nat Rev Drug Discov* 2015;**14**:475–86
16. Stevens JL, Baker TK. The future of drug safety testing: expanding the view and narrowing the focus. *Drug Discov Today* 2009;**14**:162–7
17. Aparicio S, Hidalgo M, Kung AL. Examining the utility of patient-derived xenograft mouse models. *Nat Rev Cancer* 2015;**15**:311–6
18. Hidalgo M, Bruckheimer E, Rajeshkumar NV, Garrido-Laguna I, De Oliveira E, Rubio-Viqueira B, Strawn S, Wick MJ, Martell J, Sidransky D. A pilot clinical study of treatment guided by personalized tumorgrafts in patients with advanced cancer. *Mol Cancer Ther* 2011;**10**:1311–6

19. Izumchenko E, Paz K, Ciznadija D, Sloma I, Katz A, Vasquez-Dunddel D, Ben-Zvi I, Stebbing J, McGuire W, Harris W, Maki R, Gaya A, Bedi A, Zacharoulis S, Ravi R, Wexler LH, Hoque MO, Rodriguez-Galindo C, Pass H, Peled N, Davies A, Morris R, Hidalgo M, Sidransky D. Patient-derived xenografts effectively capture responses to oncology therapy in a heterogeneous cohort of patients with solid tumors. *Ann Oncol* 2017;**28**:2595–605
20. Shekhar TM, Burvenich IJG, Harris MA, Rigopoulos A, Zanker D, Spurling A, Parker BS, Walkley CR, Scott AM, Hawkins CJ. Smac mimetics LCL161 and GDC-0152 inhibit osteosarcoma growth and metastasis in mice. *BMC Cancer* 2019;**19**:18
21. Cekanova M, Rathore K. Animal models and therapeutic molecular targets of cancer: utility and limitations. *Drug Des Devel Ther* 2014;**8**:1911-21
22. Lv D, Hu Z, Lu L, Lu H, Xu X. Three-dimensional cell culture: a powerful tool in tumor research and drug discovery. *Oncol Lett* 2017;**14**:6999–7010
23. Ham SL, Joshi R, Thakuri PS, Tavana H. Liquid-based three-dimensional tumor models for cancer research and drug discovery. *Exp Biol Med* 2016;**241**:939–54
24. Kim J, Koo B-K, Knoblich JA. Human organoids: model systems for human biology and medicine. *Nat Rev Mol Cell Biol* 2020;**21**:571–84
25. Driehuis E, Kretzschmar K, Clevers H. Establishment of patient-derived cancer organoids for drug-screening applications. *Nat Protoc* 2020;**15**:3380–409
26. Crespo M, Vilar E, Tsai S-Y, Chang K, Amin S, Srinivasan T, Zhang T, Pipalia NH, Chen HJ, Witherspoon M, Gordillo M, Xiang JZ, Maxfield FR, Lipkin S, Evans T, Chen S. Colonic organoids derived from human induced pluripotent stem cells for modeling colorectal cancer and drug testing. *Nat Med* 2017;**23**:878–84
27. Lee SH, Hu W, Matulay JT, Silva MV, Owczarek TB, Kim K, Chua CW, Barlow LJ, Kandoth C, Williams AB, Bergren SK, Pietzak EJ, Anderson CB, Benson MC, Coleman JA, Taylor BS, Abate-Shen C, McKiernan JM, Al-Ahmadie H, Solit DB, Shen MM. Tumor evolution and drug response in patient-derived organoid models of bladder cancer. *Cell* 2018;**173**:515–28.e17
28. Vlachogiannis G, Hedayat S, Vatsiou A, Jamin Y, Fernández-Mateos J, Khan K, Lampis A, Eason K, Huntingford I, Burke R, Rata M, Koh D-M, Tunariu N, Collins D, Hulkki-Wilson S, Ragulan C, Spiteri I, Moorcraft SY, Chau I, Rao S, Watkins D, Fotiadis N, Bali M, Darvish-Damavandi M, Lote H, Eltahir Z, Smyth EC, Begum R, Clarke PA, Hahne JC, Dowsett M, de Bono J, Workman P, Sadanandam A, Fassan M, Sansom OJ, Eccles S, Starling N, Braconi C, Sottoriva A, Robinson SP, Cunningham D, Valeri N. Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. *Science* 2018;**359**:920–6
29. Eduati F, Utharala R, Madhavan D, Neumann UP, Longerich T, Cramer T, Saez-Rodriguez J, Merten CA. A microfluidics platform for combinatorial drug screening on cancer biopsies. *Nat Commun* 2018;**9**:2434
30. Jeon JS, Bersini S, Gilardi M, Dubini G, Charest JL, Moretti M, Kamm RD. Human 3D vascularized organotypic microfluidic assays to study breast cancer cell extravasation. *Proc Natl Acad Sci U S A* 2015;**112**:214–9
31. Siegel R, DeSantis C, Jemal A. Colorectal cancer statistics, 2014. *CA Cancer J Clin* 2014;**64**:104–17
32. Saridaki Z, Souglakos J. Genetic alterations in colorectal cancer in older patients. *Manag Color Cancers Older People* 2013;**9**:20
33. McCullough A. Comprehensive molecular characterization of human colon and rectal cancer. *Yearb Pathol Lab Med* 2013;**2013**:295–6
34. Nandan MO, Yang VW. An update on the biology of RAS/RAF mutations in colorectal cancer. *Curr Colorectal Cancer Rep* 2011;**7**:113–20
35. Isnaldi E, Garuti A, Cirmena G, Scabini S, Rimini E, Ferrando L, Lia M, Murialdo R, Tixi L, Carminati E, Panaro A, Gallo M, Grillo F, Mastracci L, Repetto L, Fiocca R, Romairone E, Zoppoli G, Ballestrero A. Clinicopathological associations and concomitant mutations of the RAS/RAF pathway in metastatic colorectal cancer. *J Transl Med* 2019;**17**:137
36. Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC, Tecle H, Barrett SD, Bridges A, Przybranowski S, Leopold WR, Saltiel AR. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat Med* 1999;**5**:810–6
37. Corcoran RB, Ebi H, Turke AB, Coffee EM, Nishino M, Cogdill AP, Brown RD, Della Pelle P, Dias-Santagata D, Hung KE, Flaherty KT, Piris A, Wargo JA, Settleman J, Mino-Kenudson M, Engelman JA. EGFR-mediated reactivation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib. *Cancer Discov* 2012;**2**:227–35
38. Bon G, Loria R, Amoreo CA, Verdina A, Sperduti I, Mastrofrancesco A, Soddu S, Diodoro MG, Mottolese M, Todaro M, Stassi G, Milella M, De Maria R, Falcioni R. Dual targeting of HER3 and MEK may overcome HER3-dependent drug-resistance of Colon cancers. *Oncotarget* 2017;**8**:108463–79
39. Wang H, Daouti S, Li WH, Wen Y, Rizzo C, Higgins B, Packman K, Rosen N, Boylan JF, Heimbrook D, Niu H. Identification of the MEK1 (F129L) activating mutation as a potential mechanism of acquired resistance to MEK inhibition in human cancers carrying the B-Raf V600E mutation. *Cancer Res* 2011;**71**:5535–45
40. Corcoran RB, Dias-Santagata D, Bergethon K, Iafrate AJ, Settleman J, Engelman JA. BRAF gene amplification can promote acquired resistance to MEK inhibitors in cancer cells harboring the BRAF V600E mutation. *Sci Signal* 2010;**3**:ra84
41. Zhan T, Ambrosi G, Wandmacher AM, Rauscher B, Betge J, Rindtorff N, Häussler RS, Hinsenkamp I, Bamberg L, Hessling B, Müller-Decker K, Erdmann G, Burgermeister E, Ebert MP, Boutros M. MEK inhibitors activate wnt signalling and induce stem cell plasticity in colorectal cancer. *Nat Commun* 2019;**10**:1–17
42. Tavana H, Jovic A, Mosadegh B, Lee QY, Liu X, Luker KE, Luker GD, Weiss SJ, Takayama S. Nanolitre liquid patterning in aqueous environments for spatially defined reagent delivery to mammalian cells. *Nat Mater* 2009;**8**:736–41
43. Tavana H, Mosadegh B, Takayama S. Polymeric aqueous biphasic systems for non-contact cell printing on cells: engineering heterocellular embryonic stem cell niches. *Adv Mater* 2010;**22**:2628–31
44. Petrak D, Atefi E, Yin L, Chilian W, Tavana H. Automated, spatiotemporally controlled cell microprinting with polymeric aqueous biphasic system. *Biotechnol Bioeng* 2014;**111**:404–12
45. Singh S, Ray LA, Shahi Thakuri P, Tran S, Konopka MC, Luker GD, Tavana H. Organotypic breast tumor model elucidates dynamic remodeling of tumor microenvironment. *Biomaterials* 2020;**238**:119853
46. Atefi E, Fyffe D, Kaylan KB, Tavana H. Characterization of aqueous two-phase systems from volume and density measurements. *J Chem Eng Data* 2016;**61**:1531–9
47. Atefi E, Lemmo S, Fyffe D, Luker GD, Tavana H. High throughput, polymeric aqueous two-phase printing of tumor spheroids. *Adv Funct Mater* 2014;**24**:6509–15 Nov 1
48. Atefi E, Joshi R, Mann JA, Tavana H. Interfacial tension effect on cell partition in aqueous two-phase systems. *ACS Appl Mater Interfaces* 2015;**7**:21305–14
49. Atefi E, Mann JA, Tavana H. Ultralow interfacial tensions of aqueous two-phase systems measured using drop shape. *Langmuir* 2014;**30**:9691–9
50. Ham SL, Atefi E, Fyffe D, Tavana H. Robotic production of cancer cell spheroids with an aqueous two-phase system for drug testing. *J Vis Exp* 2015;**2015**:1–9
51. Shahi Thakuri P, Ham SL, Luker GD, Tavana H. Multiparametric analysis of oncology drug screening with aqueous two-phase tumor spheroids. *Mol Pharm* 2016;**13**:3724–35
52. Thakuri PS, Gupta M, Plaster M, Tavana H. Quantitative size-based analysis of tumor spheroids and responses to therapeutics. *Assay Drug Dev Technol* 2019;**17**:140–9
53. Solit DB, She Y, Lobo J, Kris MG, Scher HI, Rosen N, Sirotnak FM. Pulsatile administration of the epidermal growth factor receptor inhibitor gefitinib is significantly more effective than continuous dosing for sensitizing tumors to paclitaxel. *Clin Cancer Res* 2005;**11**:1983–9
54. Bennouna J, Sastre J, Arnold D, Österlund P, Greil R, Van Cutsem E, von Moos R, Viéitez JM, Bouché O, Borg C, Steffens C-C, Alonso-Orduña V, Schlichting C, Reyes-Rivera I, Bendahmane B, André T, Kubicka S. Continuation of bevacizumab after first progression in metastatic colorectal cancer (ML18147): a randomised phase 3 trial. *Lancet Oncol* 2013;**14**:29–37

55. Thakuri PS, Gupta M, Joshi R, Singh S, Tavana H. Synergistic inhibition of kinase pathways overcomes resistance of colorectal cancer spheroids to cyclic targeted therapies. *ACS Pharmacol Transl Sci* 2019;**2**:275–84
56. Shahi Thakuri P, Luker GD, Tavana H. Cyclical treatment of colorectal tumor spheroids induces resistance to MEK inhibitors. *Transl Oncol* 2019;**12**:404–16
57. Mirzoeva OK, Das D, Heiser LM, Bhattacharya S, Siwak D, Gendelman R, Bayani N, Wang NJ, Neve RM, Guan Y, Hu Z, Knight Z, Feiler HS, Gascard P, Parvin B, Spellman PT, Shokat KM, Wyrobek AJ, Bissell MJ, McCormick F, Kuo WL, Mills GB, Gray JW, Korn WM. Basal subtype and MAPK/ERK kinase (MEK)-phosphoinositide 3-kinase feedback signaling determine susceptibility of breast cancer cells to MEK inhibition. *Cancer Res* 2009;**69**:565–72
58. Wee S, Jagani Z, Kay XX, Loo A, Dorsch M, Yao YM, Sellers WR, Lengauer C, Stegmeier F. PI3K pathway activation mediates resistance to MEK inhibitors in KRAS mutant cancers. *Cancer Res* 2009;**69**:4286–93
59. Ham SL, Joshi R, Luker GD, Tavana H. Engineered breast cancer cell spheroids reproduce biologic properties of solid tumors. *Adv Healthc Mater* 2016;**5**:2788–98
60. Shahi Thakuri P, Tavana H. Single and combination drug screening with aqueous biphasic tumor spheroids. *SLAS Discov* 2017;**22**:507–15
61. Shahi Thakuri P, Lamichhane A, Singh S, Gupta M, Luker GD, Tavana H. Modeling adaptive resistance of KRAS mutant colorectal cancer to MAPK pathway inhibitors with a three-dimensional tumor model. *ACS Pharmacol Transl Sci* 2020;**3**:1176–87
62. Valkenburg KC, de Groot AE, Pienta KJ. Targeting the tumour stroma to improve cancer therapy. *Nat Rev Clin Oncol* 2018;**15**:366–81
63. Bergfeld SA, Blavier L, DeClerck YA. Bone marrow-derived mesenchymal stromal cells promote survival and drug resistance in tumor cells. *Mol Cancer Ther* 2014;**13**:962–75
64. Boire A, Covic L, Agarwal A, Jacques S, Sherifi S, Kuliopulos A. PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell* 2005;**120**:303–13
65. Plaster M, Singh S, Tavana H. Fibroblasts promote proliferation and matrix invasion of breast cancer cells in co-culture models. *Adv Therap* 2019;**2**:1900121
66. Roma-Rodrigues C, Mendes R, Baptista PV, Fernandes AR. Targeting tumor microenvironment for cancer therapy. *Int J Mol Sci* 2019;**20**:840
67. Joyce JA. Therapeutic targeting of the tumor microenvironment. *Cancer Cell* 2005;**7**:513–20
68. Al-Lazikani B, Banerji U, Workman P. Combinatorial drug therapy for cancer in the post-genomic era. *Nat Biotechnol* 2012;**30**:679–92
69. Jin M-Z, Jin W-L. The updated landscape of tumor microenvironment and drug repurposing. *Signal Transduct Target Ther* 2020;**5**:166
70. Palmer AC, Sorger PK. Combination cancer therapy can confer benefit via patient-to-patient variability without drug additivity or synergy. *Cell* 2017;**171**:1678–91
71. Bedard PL, Tabernero J, Janku F, Wainberg ZA, Paz-Ares L, Vansteenkiste J, Van Cutsem E, Pérez-García J, Stathis A, Britten CD, Le N, Carter K, Demanse D, Csonka D, Peters M, Zubel A, Nauwelaerts H, Sessa C. A phase Ib dose-escalation study of the oral pan-PI3K inhibitor buparlisib (BKM120) in combination with the oral MEK1/2 inhibitor trametinib (GSK1120212) in patients with selected advanced solid tumors. *Clin Cancer Res* 2015;**21**:730–8
72. Wainberg ZA, Alsina M, Soares HP, Braña I, Britten CD, Del Conte G, Ezeh P, Houk B, Kern KA, Leong S, Pathan N, Pierce KJ, Siu LL, Vermette J, Tabernero J. A multi-arm phase I study of the PI3K/mTOR inhibitors PF-04691502 and gedatolisib (PF-05212384) plus irinotecan or the MEK inhibitor PD-0325901 in advanced cancer. *Target Oncol* 2017;**12**:775–85
73. Shimizu T, Tolcher AW, Papadopoulos KP, Beeram M, Rasco DW, Smith LS, Gunn S, Smetzer L, Mays TA, Kaiser B, Wick MJ, Alvarez C, Cavazos A, Mangold GL, Patnaik A. The clinical effect of the dual-targeting strategy involving PI3K/AKT/mTOR and RAS/MEK/ERK pathways in patients with advanced cancer. *Clin Cancer Res* 2012;**18**:2316–25