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Annu Rev Cancer Biol. Author manuscript; available in PMC 2022 March 01.

Published in final edited form as:

Annu Rev Cancer Biol. 2021 March ; 5: 39–57. doi:10.1146/annurev-cancerbio-043020-125955.

Ex Vivo Analysis of Primary Tumor Specimens for Evaluation of Cancer Therapeutics

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Abstract

The use of *ex vivo* drug sensitivity testing to predict drug activity in individual patients has been actively explored for almost 50 years without delivering a generally useful predictive capability. However, extended failure should not be an indicator of futility. This is especially true in cancer research where ultimate success is often preceded by less successful attempts. For example, both immune- and genetic-based targeted therapies for cancer underwent numerous failed attempts before biological understanding, improved targets, and optimized drug development matured to facilitate an arsenal of transformational drugs. Similarly, the concept of directly assessing drug sensitivity of primary tumor biopsies—and the use of this information to help direct therapeutic approaches—has a long history with a definitive learning curve. In this review, we will survey the history of *ex vivo* testing as well as the current state of the art for this field. We will present an update on methodologies and approaches, describe the use of these technologies to test cutting-edge drug classes, and describe an increasingly nuanced understanding of tumor types and models for which this strategy is most likely to succeed. We will consider the relative strengths and weaknesses of predicting drug activity across the broad biological context of cancer patients and tumor types. This will include an analysis of the potential for *ex vivo* drug sensitivity testing to accurately predict drug activity within each of the biological hallmarks of cancer pathogenesis.

Introduction

The use of *ex vivo* drug sensitivity assays as diagnostic tools for clinical therapeutic decision making has an extensive and, sometimes, checkered history. This has driven some to conclude that the effort is futile, however, more recent approaches are showing substantial

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promise. Improved methodologies, readouts, and classes of drugs that can be tested have led to encouraging results as well as an expansion of the ways in which this concept can be used. In addition to the idea of using these assays to aid in clinical decision making for individual patients, more recent studies have also leveraged large data sets that integrate *ex vivo* drug sensitivity data with other data types, such as genomic, transcriptomic, and clinical information, to enable insights into biological mechanisms of drug response and identification of drug sensitivity signals that may have been missed without these agnostic approaches. Additionally, retrospective studies of contemporary classes of drugs tested with newer drug sensitivity assay technologies have repeatedly demonstrated high predictive power for clinical response. In the context of renewed excitement around the field of *ex vivo* drug sensitivity testing (including the formation of the Society for Functional Precision Medicine, SFPM, https://www.sfpm.io), it is worth revisiting the history of this field to further understand how updated approaches have facilitated improved use of these assays. It is also worth considering the domains of cancer biology and classes of therapeutics that are best suited for this type of testing and the areas where current technologies still may face challenges in accurately predicting drug activity. This can perhaps best be conceptualized by taking into consideration the capacity for current approaches to accurately model each of the biological hallmarks of cancer.

Historical Perspective

The concept of using *ex vivo* functional testing of primary tumor biopsies to evaluate and prioritize drug sensitivities has existed for at least 50 years. Over the past ~20 years, many have formed an opinion that this approach is not successful, and this has been at least partly driven by working group literature reviews(Burstein et al 2011, Samson et al 2004, Schrag et al 2004). It is worth revisiting these initial studies, the methodologies that were employed, tumor types that were studied, drugs that were evaluated, and the actual performance of the assays in those studies.

Assay readouts

A number of methodologies have been employed for *ex vivo* drug sensitivity testing. Early studies involved cellular disassociation of tumor biopsies, exposure of unselected cells to a single concentration of each drug for a short period (typically 1 hour), washing of the cells and plating into a semi-solid media. After 2 weeks, cells that expanded to form colonies were manually enumerated with brightfield microscopy(Salmon et al 1978, Von Hoff et al 1983, Von Hoff et al 1991, Von Hoff et al 1990). In other cases, single-cell suspensions from disassociated tumors were placed into culture and efficacy of a small panel of drugs was evaluated by dye exclusion assays and manual enumeration of viable cells in drug-treated versus control conditions(Brower et al 2008, Cortazar et al 1997, Gallion et al 2006, Gazdar et al 1990, Mi et al 2008, Shaw et al 1993, Wilbur et al 1992). Typically, this required expansion of the initial tumor biopsy, which occurred over ~1–12 weeks in culture. Importantly, due to the timing of such cell line expansion assays, clinical implementation of results was not typically executed at the clinical stage of tumor biopsy acquisition, but only at the time of a subsequent disease relapse. In addition, as expanded on below, the drugs

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tested in these early studies were not targeted to molecular abnormalities of the tumors, hence, would have been less likely to strongly determine response.

A number of variations on these methods have been employed, such as more automated measurements of cell viability through use of ATP content quantification from lysed cells(Cree et al 2007, Kurbacher et al 1998, Ugurel et al 2006) or assessment of cell metabolic activity (MTT) at the assay endpoint(Wu et al 2008, Xu et al 1999) as well as the use of tritiated thymidine to measure proliferative cells in semi-solid media(Joo et al 2009, Kern et al 1985, Kern & Weisenthal 1990, Loizzi et al 2003). Some studies used alternative, *in vivo* assays such as surgical implantation of tumor pieces into kidneys of immunocompetent CD_2F_1 mice and excision of tumors after short in vivo treatment courses (~5 days)(Maenpaa et al 1995).

Characterization of Assayed Cells

One major issue for *ex vivo* drug sensitivity testing has been the characterization of the extent to which tumor cells maintain a variety of biological properties found in the original tumor. The technical capacity to perform detailed validation studies of these *ex vivo* cells was often limited in earlier studies. In many cases, validation of tumor fidelity was limited to cytologic/morphologic stains (e.g. Hematoxylin/Eosin, Wright-Giemsa, Papanicolaou, peroxidase, dye exclusion, etc.)(Brower et al 2008, Cortazar et al 1997, Gallion et al 2006, Gazdar et al 1990, Mi et al 2008, Shaw et al 1993, Wilbur et al 1992), the capacity for culture surfaces, such as polypropylene(Wilbur et al 1992), or media/suspension formats, such as soft agar to exclude non-tumor cell growth(Joo et al 2009, Kern et al 1985, Kern & Weisenthal 1990, Loizzi et al 2003, Von Hoff et al 1991, Von Hoff et al 1990), and the favoring of tumor versus non-tumor cells for *in vivo* growth(Maenpaa et al 1995). Initial validation studies had been performed for some assays using microscopy, karyotyping, cytologic stains, or clonogenicity of cells in immunocompromised mice (summarized in(Von Hoff et al 1983)). However, most of these readouts would not be considered sufficient by current standards to validate tumor fidelity, and we now know that many of these validation techniques do not truly distinguish tumor from non-tumor cells. Often, these studies were carried out only for initial assay development, but not performed for the cases being tested for actual clinical decision-making (or only for a small fraction, such as 5% of cases(Von Hoff et al 1983)). Clearly, as this field has moved forward, the utilization of technological improvements in cellular and molecular biology to facilitate rigorous and high-throughput validation of *ex vivo* tumor cell biology—from the standpoint of genetics, cell state, and heterogeneity of cell populations—has been important.

Tumor Types

Initial studies in the field of ex vivo drug sensitivity testing were confined to a relatively small number of solid tumor types – primarily breast(Brower et al 2008, Kern et al 1985, Kern & Weisenthal 1990, Mi et al 2008, Von Hoff et al 1983, Von Hoff et al 1990, Xu et al 1999), ovarian(Cree et al 2007, Gallion et al 2006, Joo et al 2009, Kurbacher et al 1998, Loizzi et al 2003, Maenpaa et al 1995, Salmon et al 1978, Von Hoff et al 1983, Von Hoff et al 1991, Von Hoff et al 1990), lung (non-small cell and small cell)(Cortazar et al 1997, Gazdar et al 1990, Kern et al 1985, Kern & Weisenthal 1990, Shaw et al 1993, Von Hoff et

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al 1983, Von Hoff et al 1990, Wilbur et al 1992), melanoma(Kern et al 1985, Kern & Weisenthal 1990, Ugurel et al 2006, Von Hoff et al 1983, Von Hoff et al 1990), and colorectal(Kern et al 1985, Kern & Weisenthal 1990, Von Hoff et al 1983, Von Hoff et al 1990, Wu et al 2008) cancer, with a small number of cases of other tumor types. Many tumor types were not tested in significant numbers in any of these studies due to a lack of success of long-term culture of these cells. This included most subsets of hematologic malignancy, although some of the earliest studies did focus on multiple myeloma(Salmon et al 1978). Since these initial studies, it has become clear that successful culture of malignant cells that maintain fidelity to the original tumor requires substantially different approaches and culture conditions from one tumor lineage to another. In addition, revised protocols favoring shorter term assays that do not involve expansion of tumor cells, media additives that ensure better maintenance of tumor cell biology, and strategies that enable retention or add back of microenvironmental tumor features, have facilitated new assay formats and enabled inclusion of previously excluded tumor types.

Furthermore, many studies tested only metastatic lesions and, in these instances, the studies typically tested a single metastatic lesion. In the intervening period since the performance of these initial studies, we have developed a much clearer understanding of the clonal evolution of tumors between primary and metastatic sites, and we also now have a fuller understanding of the biological divergence that occurs between distinct metastatic sites. With this knowledge in mind, it is probably unsurprising that an *ex vivo* drug sensitivity test on a single metastatic lesion was not always predictive for overall response of the patient.

Drugs Tested and Definitions of Drug Activity

Importantly, the drugs that were tested in these early studies were relatively few in number and were restricted to classes of chemotherapy drugs with broad toxicity profiles. Drug classes tested included RNA polymerase inhibitors, alkylating agents, DNA-damaging agents, topoisomerase inhibitors, antimetabolites, and microtubule-disruptive drugs. These drugs rarely elicit durable responses in patients with advanced disease (the populations being tested in these studies), and the window of response between most sensitive and least sensitive cases was narrow, creating difficulties in distinguishing and clinically correlating exceptional responses. Many newer classes of agents were not yet available for testing at the time of these studies, and as will be covered later in this review, it is becoming clear that not all drug classes can be equally assessed using *ex vivo* drug sensitivity testing – indeed the format for the assay must be considered in the context of the mechanism of action of the drug (discussed below in greater detail).

The definition for activity of a drug is also an important consideration. One approach involved assays that focused on extreme drug resistance (EDR) to identify therapeutic strategies unlikely to provide clinical benefit(Eltabbakh et al 1998, Joo et al 2009, Kern 1998, Mehta et al 2001, Nagourney 2000, Soslow et al 2000). These EDR assays have seen extensive clinical use and include formats that have been commercialized. However, most studies focused on identification of sensitive cases and in many initial studies, the definition of an active drug was a reduction of 50% tumor cell growth compared with control conditions. Although some studies attempted to rank order drug activity, many studies

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considered drugs to be active once they surpassed this 50% threshold. This presents one key question that still confronts the field today – how best to interpret *ex vivo* drug activity in a way that will most accurately translate into clinical activity.

Reported Activity

In spite of all of the technical challenges faced by early iterations of *ex vivo* drug sensitivity testing, there were signals in most studies that response rates were significantly improved in the drug sensitivity-guided arm. Improvement in response to drug treatment was typically transient, which most likely represented the best possible outcome for many of the cases tested for the given drug regimens utilized. However, these results pointed to an initial promising concept that has since been improved with more advanced readouts, the advent of newer drug classes, greater rigor in characterizing cellular inputs and outputs, and careful consideration of the biology being modeled.

In this respect, it is worth revisiting the potential of *ex vivo* drug sensitivity testing. Early conclusions that these approaches were not yet suitable for wide-scale implementation in their current state was certainly warranted. However, conjecture that these approaches cannot work seems premature, especially given the technical advances seen in current approaches that have facilitated improvements in both rigor and biological knowledge.

The Present: Current State of Assay Development

Current approaches to *ex vivo* testing of drug sensitivity have evolved to include numerous formats across different tumor types. These more advanced platforms harness the use of distinct media formulations, testing in two- as well as three-dimensions, inclusion of multiple cell types, various readouts for measuring phenotypic responses in aggregate cell populations as well as in single-cells, measurements of drug impact on specific cellular pathways and processes, and *in vivo* approaches. A review in 2015(Friedman et al 2015) provided an excellent overview of many of these techniques. Here, we provide an updated view and consider the relative merits of these approaches for accurately assessing drug activity within each biological hallmark of cancer. This will include discussion of the path forward for implementation of and improvements to the field of *ex vivo* drug sensitivity testing.

Direct Phenotypic Measurements

Many different approaches have built upon the early strategies described above, and have made important changes to assay format and readout. At a basic level, there are still similarities in approach – tumor biopsies (liquid or solid) are obtained from patients, and cells are exposed to drugs with readout of functional impact of drugs on cell phenotype. However, many efforts have been made to shorten the time to assay completion which enables testing on non-expanded cells, thereby eliminating the chance for tumor cells to drift and/or non-tumor cells to overtake the culture. Furthermore, additional updating of culturing conditions and implementation of cutting-edge engineering technologies have also helped to better mimic the native tumor microenvironment.

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Two Dimensions

Two-dimensional cultures have taken on numerous formats over the past 10–15 years. These have included strategies for both hematologic and solid tumors and formats have diverged between the tumor types. For hematologic malignancies, two-dimensional liquid cultures have most commonly been performed in “basic” media conditions that forgo most media supplements in favor of minimal essential components to maintain cell viability, either in the form of fetal bovine serum(Tyner et al 2013) or minimal mixtures of recombinant protein supplements(Pemovska et al 2013). This has been done in an attempt to protect the cell’s phenotypic responses from undue influence from external stimuli and preserve drug responses that are most determined by cellular intrinsic features of the tumor.

Alternative methods have incorporated exogenous growth factors to maintain higher cell viability and/or recapitulate certain elements of the tumor microenvironment over the course of short-term culture. These factors are sometimes derived from conditioned media of stromal cells or from recombinant proteins. In certain cases, rigorous comparisons have been made between these different media conditions. Results have shown that careful selection of media conditions can improve efficiency of successful culture for certain cancer types, such as ovarian tumors, as well as retaining the molecular and histopathologic landscape of the original tumor in long-term culture(Ince et al 2015). Other studies have used this approach to model the impact on drug response of a matrix of microenviornmental factors ((Watson et al 2018) and other similar examples described in more detail below). In studies of hematologic cancers that have tested different media compositions, data have indicated that many classes of drugs exhibited similar results between the different conditions, however, substantial differences in activity were seen with certain drug classes with some drug families, such as topoisomerase inhibitors, BCL2 inhibitors, and many kinase inhibitors, that exhibited better tumor cell killing in basic conditions versus JAK kinase inhibitors, that were more active in supplemented conditions(Karjalainen et al 2017). Changes in basic biology, such as changes in BCL2 family member usage and dependence were also observed from one media condition to another. These findings point to a very important concept for the field of *ex vivo* drug sensitivity testing – different classes of drugs may require customized strategies for testing to ensure results that are most indicative of *in vivo* biology and predictive of clinical response, and emphasize the need to capture the related *in vivo* biology and patient response.

Alternative media supplements have also been shown to promote the length of time that primary leukemia cells can be maintained *ex vivo* without undergoing spontaneous differentiation. Indeed, the functional determinants of hematopoietic stem cell (HSC) self-renewal play an important role in *ex vivo* culturing and screening of primary hematopoietic cells. Key regulators of HSC self-renewal, such as JARID1B, JHDMLF, MSI2, and PROX1, influence hematopoietic stem cell renewal in both mouse and human *ex vivo* model systems(Cellot et al 2013, Deneault et al 2009, Hope et al 2010). A number of studies have demonstrated that antagonists of aryl hydrocarbon receptors (AHR) such as SR1-4(Boitano et al 2010, Bouchez et al 2011) and agonists of NOTCH ligand(Delaney et al 2010) have the ability to support expansion of human long-term HSCs *in vivo* and *in vitro*. High-throughput chemical screens helped identify small molecules, such as UM171 and UM729, with the

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ability to synergize with AHR antagonists in inhibiting leukemia stem cell (LSC) differentiation and supporting LSC activity in vitro(Fares et al 2014, Pabst et al 2014). The mechanism of action of UM171 is thought to be through effects on pro- and anti-inflammatory and detoxification networks. These networks mediate self-renewal and support hematopoietic stem cell (HSC) expansion, which are controlled by NFKB activation and protein C receptor (PROCR)-dependent reactive oxygen species (ROS) detoxification(Chagraoui et al 2019). Using a shRNA library screen, MacPherson and colleagues identified members of the HBO1 protein complex that were also critical regulators of LSC maintenance(MacPherson et al 2020). They developed a highly potent small molecule inhibitor of HBO1 and demonstrated that the compound was a competitive analogue of acetyl-CoA.

For solid tumors, different media conditions have been determined to facilitate expansion of solid tumor cells in a way that maintains good fidelity of both genetic and cell state. This strategy has been termed "conditional reprogramming" and involves co-culture of tumor biopsy cells on a feeder layer of irradiated fibroblasts as well as the addition of small-molecule inhibitors of RHO kinase (ROCK). Recent iterations of this demonstrate that some tumor types don't require the fibroblast feeder layer and that the key to continued proliferation is the ROCK inhibitor. These updated media conditions, combined with miniaturization strategies have facilitated limited expansion of primary solid tumor cells that remain comparable to the original tumor and can also enable testing of much larger panels of agents, including the newest classes of drugs. Importantly, patient cells propagated with these growth conditions have been validated against original tumor tissue by short tandem repeat profiling, comparative genomic hybridization, and spectral karyotyping(Liu et al 2017, Liu et al 2012, Suprynowicz et al 2012, Yuan et al 2012). Additional approaches not dependent on a ROCK inhibitor have been identified for a number of tumor lineages that also retain tumor fidelity. Indeed, similar to hematologic malignancies, there is an expanding literature base on diverse culture conditions to propagate a variety of solid tumor types (for example(Ince et al 2015)), indicating a need to evaluate the strategies that are most successful at recapitulating the *in vivo* tumor biology and clinical response. Strategies that can retain or enable adding back of microenvironmental features of in vivo tumor biology might be expected to exhibit the best predictive power.

In addition to the use of two-dimensional cultures as a tool to understand drug sensitivity at the level of individual patient samples and drugs, many studies have taken a population perspective to understand the patterns of sensitivity across broad panels of drugs and the manner by which these sensitivity patterns correlate with genetic, transcriptomic, and clinical features in hematologic malignancies(Dietrich et al 2018, Frismantas et al 2017, Lee et al 2018, Pemovska et al 2013, Tyner et al 2018, Tyner et al 2013). Studies of a similar nature in solid tumors have also incorporated a combination of two-dimensional, three-dimensional, and *in vivo* patient-derived models to integrate with genomics across a variety of solid tumor types(Brodin et al 2019, Friedman et al 2017, Pauli et al 2017, Saeed et al 2019, Saeed et al 2017), and in some cases sensitivity to RNAi-mediated gene knockdown has been included in the analyses to help understand and prioritize drug sensitivity patterns(Moser et al 2014, Tyner et al 2009, Xu et al 2018). Analytical tools to assist with such studies, such as the open source Breeze platform for quality control and analysis of

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high-throughput drug sensitivity data, will assuredly help with these large-scale analyses in future studies(Potdar et al 2020).

Three-Dimensional Semi-Solid Cultures

In 1966, Bradley and Metcalf developed the first ex vivo assay performed on murine hematopoietic cell isolates which was able to assess the number of the colony forming units (CFU) or colony forming cells (CFC) in a given sample. This clonogenic assay, which measures the differentiation and maturation of CFCs helped to characterize the granulocyte-macrophage lineage (GM-CFU) and also identified a key growth factor called colony stimulating factor (CSF)(Bradley & Metcalf 1966). Pike and Robinson were able to adopt the assay for growing human bone marrow cell colonies in agar-gel medium(Pike & Robinson 1970) and Norman Iscove developed a second-generation technique that utilized methylcellulose(Iscove et al 1974) with or without supplemental growth factors. This assay is still used extensively today, and the development of these *ex vivo* bone marrow colony formation assays has been instrumental for understanding normal hematopoiesis as well as for assessment of leukemia cell clonogenic potential. While colony formation assays have been used to assess the impact of drug exposure on leukemia cell growth and clonogenicity, this technique has not readily been transformed into a high throughput method for drug sensitivity assessment.

A conceptually related technique for expansion and testing of solid tumor cells in semi-solid media – organoids – has been broadly used to expand solid tumor cells *ex vivo* for many different experimental purposes, including drug sensitivity evaluation. Following dissociation of primary biopsies, cells are plated into polymerized Matrigel with a variety of recombinant and small-molecule additives, including agonists of EGF and WNT signaling and inhibitors of TGFB and ROCK pathways(Sato et al 2011, Sato et al 2009). Alternative approaches have included culture of cells in collagen matrices that incorporate an air-liquid interface with some of the same culture additives, such as WNT pathway agonists(Ootani et al 2009). Heterogeneous cell mixtures, such as tumor cells with fibroblasts have been tested in this air-liquid interface system to show changes on drug sensitivity with cell co-cultures compared with tumor-only organoids(Prina-Mello et al 2018). Organoid technology has also been miniaturized, taking advantage of a simplified geometry that seeds cells around the rim of the wells (mini-rings), and this has facilitated more automated, high-throughput screening of larger collections of compounds in a shorter, more clinically-relevant timeframe(Phan et al 2019). Organoid protocols, with numerous modifications have been used to study normal developmental biology of numerous tissue types, to generate genetically engineered *in vitro* models that recapitulate common genetic lesions, such as mutation of KRAS or loss of TP53, APC, and SMAD4, and to establish organoid cultures from primary tumors derived from the head and neck, pancreas, breast, colorectal, and rectal regions. Recent reviews of the organoid field have summarized these advances (Clevers & Tuveson 2019, Neal & Kuo 2016, Tuveson & Clevers 2019).

Some of the studies that derived organoids from primary tumors have provided evidence that non-tumor cell types, such as tumor-infiltrating immune cells and cancer-associated fibroblasts, can be maintained within the expanded organoids. These multi-cellular models

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are able to recapitulate certain elements of the tumor microenvironment and can facilitate studying the impact of perturbations on the tumor microenvironment as well as the tumor cells(Neal et al 2018). These findings have been mirrored in some of the hematopoietic primary culture systems, where non-tumor cells such as macrophages and T-cells have been shown to play an important influencing role in drug responses and have facilitated the *ex vivo* analysis of additional classes of agents, such as those that act on immune cells(Carey et al 2017, Edwards et al 2019, Edwards et al 2018, Kuusanmaki et al 2020, Majumder et al 2019). A related approach leveraged microarray technology to create a matrix of pairwise combinations of recombinant extracellular matrix proteins with soluble growth factors and cytokines(Smith et al 2019) and showed some of these factors could influence breast cancer cell response to tyrosine kinase inhibitors in a disease subset specific way(Watson et al 2018). While these findings have collectively opened up a new avenue of research for *ex vivo* tumor models, they have also created a need for updated readouts that incorporate single-cell granularity and multi-parameter phenotypes. This increase in sophistication of readouts will facilitate both the distinguishing of tumor from non-tumor cells in scoring response, and will also allow for scoring of desirable phenotypic impacts on the non-tumor cells (e.g. promotion of T-cell activation states). Furthermore, as with two-dimensional culture, different formulations of media components are under investigation to determine if additional phenotypic states and tumor cell heterogeneity reflective of the parent tumor can be obtained in organoid cultures.

Induced pluripotent stem cells (iPSC) have also been used in conjunction with organoid techniques to produce disease models from cancers such as colorectal carcinoma that exhibit organoid architecture, cellular composition, and pathway activation states that maintain high fidelity to the biology of the *in vivo* tumor state(Crespo et al 2017). A variety of other tissue types have been used in conjunction with iPSC technology to derive high fidelity models (reviewed in(Papapetrou 2016)), and these techniques promise to offer robust model systems for future *ex vivo* cancer drug screening.

3D Bio-printed models and Organotypic Cultures

In some of the most recent strategies, three-dimensional bio-printing technologies have been integrated with primary cell and organoid techniques to add a variety of biologically important features to primary patient *ex vivo* models. These approaches have enabled the creation of spatial architecture and physical properties, such as fluid flow, that resemble the native tumor microenvironment. Testing has been performed on media, or bioink, with varying levels of components such as gelatin and alginate to show that different bioink formulations can impact on bio-printed tissue phenotypes(Jiang et al 2019).

Additional components, such as laminin/peptide additives to matrigel(Schmidt et al 2019), as well as infiltrating cell types, such as fibroblasts(Amann et al 2014), have been incorporated into semi-solid matrices to interact with tumor cells in a way that mimics the *in vivo* biology of the tumor. These approaches have been used to create *ex vivo* niches that resemble tumor microenvironments. In some cases, these strategies have integrated cellular components from multiple organs, such as liver, heart, and lung(Skardal et al 2017). The bone marrow microenvironment of hematologic malignancies has also been modeled in

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three-dimensional scaffolds made of components such as fibrin gels or zirconium oxide and containing purified cells such as mesenchymal stromal cells and/or hematopoietic stem progenitor cells that were shown to preserve a population of HSPCs giving rise to multi-lineage differentiated blood cells(Chou et al 2020, Sieber et al 2018). In one recent study, a multi-cellular lung organoid that exhibited air sac structures, production of lung surfactant protein, and angiogenesis was used in a multi-cellular organoid platform to mimic lung colonization of tumor cells, and drugs that impact on metastasis and angiogenesis were successfully tested in this assay(Ramamoorthy et al 2019). Another study demonstrated scaffold-free bio-printing with defined architecture to model tumor-stromal interactions, including multiple stromal cell types, native ECM deposition, and self-organized vasculature, which allowed evaluation of tumor phenotypes including proliferation, signaling and migration in response to extrinsic signals and therapies(Langer et al 2019). Reviews of these three-dimensional bio-printing techniques have been recently published(Ashok et al 2020, Brancato et al 2020, Fan et al 2019, Schneeberger et al 2017).

Related approaches have also been developed to more closely model the *in vivo* tumor microenvironment by culturing thin slices of tumor biopsies in their native state (organotypic cultures), which have enabled retention of native states of cellular heterogeneity, spatial architecture, and have shown high rates of predictivity of clinical responses(Hirt et al 2014, Kenny et al 2015, Majumder et al 2015, Nagourney et al 2012, Ridky et al 2010, Vaira et al 2010).

Evolving Readouts

Over the history of *ex vivo* drug sensitivity testing, readouts have evolved dramatically. As described above, initial strategies required laborious and subjective manual quantification under a microscope, which later gave way to automated strategies that measure thymidine incorporation, ATP content, or metabolic activity of cells. In recent years, new instrumentation and biological tools have facilitated dramatic updates to *ex vivo* readouts. These include the incorporation of high-throughput flow cytometry(Kuusanmaki et al 2020, Majumder et al 2019, Teh et al 2020) and imaging analysis(Jacob et al 2016), both of which enable quantification of specific phenotypes on a single-cell level. These new techniques can then be combined with the multitude of high-quality antibodies that are now available to organize cells into discrete biological populations as well as antibodies that can accurately distinguish between specific cell states (proliferation, death, activation) and/or measure drug impact on specific pathways (signaling, apoptosis, etc.). Together they have enabled the identification of cell sub-populations that demonstrate pathway resistance to drug exposure and have facilitated important discoveries, such as the manner by which certain drugs and drug classes act preferentially on cells from specific lineages (both healthy and malignant), while sparing others that can give rise to resistance and relapse. These assays also offer the possibility of assessing the effects of specific interactions (e.g. tumor-fibroblast or tumor-immune) on drug response.

Single cell analyses have also been applied in a number of formats involving microfluidic chips measuring a variety of readouts including buoyant mass of cells. These techniques can reveal single-cell heterogeneity of bulk tumor cells and can be used as a readout of viability

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of single cells from tumors (bulk hematopoietic tumors or circulating tumor cells (CTSs) from solid tumors) after exposure to therapeutic agents. Modified media and culturing conditions, such as suspension culture to mimic the biology of circulating cells, has been shown to improve readouts from CTCs. Other groups have also harvested circulating tumor cells (CTCs) from patient blood and established cultures of these CTCs that have enabled drug sensitivity assessments(Brouzes et al 2009, Stevens et al 2016, Yu et al 2014).

Other new technologies have also facilitated the direct measurement of pathway states, which have included pathways critical to apoptosis. One prominent method that has been used in this way has been BH3 profiling, which measures the propensity of cells to undergo apoptosis in a manner that distinguishes dependence on specific BCL2 family members(Certo et al 2006, Deng et al 2007, Letai 2008). This technique can be done for cells in a static or dynamic state(Montero & Letai 2016, Montero et al 2015) and has been modified to be performed in aggregate with plate-reader technology or in single cells with flow cytometry(Ryan & Letai 2013). BH3 profiling has been used to successfully predict response to a wide variety of cancer therapeutics and tumor types, and it was a pivotal technique for the successful development and implementation of inhibitors of BCL2 family members for lymphoid and myeloid leukemias(Del Gaizo Moore et al 2007, Deng et al 2007, Konopleva et al 2016, Pan et al 2014, Touzeau et al 2016).

In vivo models

A variety of in vivo models have also been utilized to functionally assess drug response in a way that will lead to new biological understanding and clinically predictive therapeutic strategies. One approach has been to utilize immunocompromised(Hudson et al 1998) mice as a vessel to expand primary tumor cells and interrogate response to in vivo dosing (reviewed in(Hidalgo et al 2014, Lai et al 2017, Meyerrose et al 2003, Shultz et al 2007)). A variety of mouse strains now exist that are immuno-deficient due to specific genetic mechanisms, leading to a range of immune compromised states. In addition, some of these strains have been engineered to express humanized components of the tumor microenvironment, such as expression of specific human growth factors and cytokines that help support engraftment and growth of specific cellular lineages(Feuring-Buske et al 2003, Ito et al 2002). Injection of tumor cells into these mice has also been done in varying formats, ranging from sub-cutaneous, intravenous or intrafemoral injections for hematologic malignancies or to model bone metastases(Wu et al 1998), injection into sub-cutaneous ossicles with humanized bone marrow microenvironment(Reinisch et al 2016), and orthotopic injections (reviewed in(Hoffman 2015)). Cumulatively, these different mouse strains and injection protocols have enabled patient-derived xenografts (PDXs) to model a wide variety of tumor types and a multitude of different drug classes and drug combinations have been tested using this approach. However, the throughput of using patient-derived xenograft models for assessment of drug sensitivity is far less than any of the ex vivo approaches described above and some studies have used these high-fidelity models to support expanded screening *ex vivo*, thereby, revealing important drug sensitivity vulnerabilities in disease of high unmet medical need, such as TCF3-HLF rearranged acute lymphoblastic leukemia(Fischer et al 2015). Further not all tumors adapt to in vivo growth in murine models. Indeed, the ability to grow as PDXs correlates with tumor aggressiveness.

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Unfortunately, due to the aggressiveness of the tumors many patients are deceased prior to being able to perform predictive drug screens(McAuliffe et al 2015).

In an effort to increase the throughput of in vivo drug assessment testing, and also to perform such testing in tumor native environments, alternative approaches have been developed to inject capsules containing segregated libraries of drugs directly into primary tumors in immune competent or compromised mouse models. Micro-dosing technology limits the spread of each drug to the immediate local region, which can be measured along with assessment of drug impact on tumor cell phenotypes upon resection of the tumor and immuno-staining of the region surrounding the injected capsule(Jonas et al 2015, Klinghoffer et al 2015). This technology has been successfully demonstrated in primary tumors in mice, and has been shown to reveal tumor adaptation(Jonas et al 2016) and impact on immune microenvironment(Frazier et al 2017). Clinical trials in humans are currently ongoing.

Hallmarks of *Ex Vivo* Drug Testing – Future Directions

Hanahan, Weinberg, and Coussens contributed foundational thought pieces around essential biological hallmarks of cancer(Hanahan & Coussens 2012, Hanahan & Weinberg 2011). These biological driver categories provide a useful framework from which to consider the various formats of *ex vivo* drug sensitivity testing, and the strategies that are best suited to evaluate drug sensitivity within each hallmark (Figure 1). It is useful to group these ten hallmarks into four categories based on the feasibility of assessing activity of drugs targeting these processes by various *ex vivo* platforms. In particular, it is key to note that capturing of some of these hallmarks involve the measurement of tumor cell phenotypes, while others depend on heterogenous cell culture systems with phenotypic measurement of non-tumor cells, such as stromal cells and/or immune cells. Strategies that can accurately model both tumor intrinsic and diverse aspects of microenvironmental biology will offer powerful tools for understanding complex interactions between drugs that target these various aspects of tumor biology.

Category 1: (1) Proliferation, (2) Anti-Apoptosis, & (3) Growth Suppression

Drugs targeting cellular proliferation (e.g. tyrosine kinase inhibitors), pathways that regulate apoptosis (e.g. BCL2 family inhibitors), and checkpoints on cell division (e.g. cyclin-dependent kinase inhibitors) have been shown to read out effectively in all formats for *ex vivo* drug sensitivity screening. Since these drugs target the cellular phenotypes that are most directly assessed with techniques that measure relative numbers of viable cells and there are also accurate biomarkers for these pathways and cellular processes, these hallmarks can dependably be measured using *ex vivo* drug sensitivity testing. Indeed, there are many examples of *ex vivo* platforms that have accurately measured sensitivity of these classes of drugs within clinical or genetic subsets with known response patterns, in retrospective correlative studies, and in growing numbers of prospective case studies. It is worth noting that pathways governing cell growth and death can be modulated by cell extrinsic signals from the microenvironment. In this respect, some of the approaches summarized below in

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category 3 may also be useful for ensuring the most accurate measurements of these category 1 hallmarks.

Category 2: (4) Metabolism, (5) Replicative Immortality & (6) Genomic Instability

The hallmarks of metabolism, replicative immortality, and genomic instability can be targeted by drugs, which impact directly on phenotypes of tumor cells that can be directly measured. However, the specific phenotypes are often not as easily or quickly measured through quantification of viable cells. In this way, these three hallmarks are also well assessed by *ex vivo* drug sensitivity assays, though, the readouts that are needed to accurately quantify drug activity may be more nuanced and, in some cases, may require biomarkers that are tailored to the specific hallmark (or specific drug). For example, metabolic inhibitors may induce differentiation rather than cell death, requiring markers of cell maturation state and assays to measure metabolic states or directly measure metabolites could be very useful for this hallmark and drug class. Drugs that target replicative immortality or genomic instability may be measured by cell death/viability more easily, though the time-frame to this endpoint may require culturing methods that can maintain cells in a proliferative state for longer periods of time.

Category 3: (7) Immune Suppression & (8) Inflammation

The assessment of immune suppression or inflammation in primary tumors has been attempted in several *ex vivo* assays. Clearly, the assays must retain a heterogeneity of cells for these platforms to be effective and, in many cases, the readouts will need to be uniquely tailored to the mode of action of the drugs (e.g. discrete measurements of T-cell activity for immune checkpoint inhibitors; assessment of secreted or cell contact-mediated factors from cancer-associated fibroblasts, etc.). Immune, inflammatory, and stromal cells are frequently lost or diluted through sequential passage making early *ex vivo* analysis of these processes important, or necessitating a means by which immune or other stromal cells can be re-introduced into *ex vivo* models, such as with bio-printed tissues.

Category 4: (9) Invasion/Metastasis & (10) Angiogenesis

The hallmarks of invasion/metastasis and angiogenesis are probably the most challenging to readout for *ex vivo* drug sensitivity assays, and most platforms are probably not capable of accurately assessing these facets of tumor biology, although recent studies of engineered tissues have made great progress in modeling these facets of tumor biology(Langer et al 2019, Ramamoorthy et al 2019). Clearly, these hallmarks can be measured using some of the *in vivo* models touched on in this review, such as patient-derived xenograft or other mouse models. Much exciting work remains to model these hallmarks using some of the more advanced, engineered three-dimensional assay technologies.

Acknowledgements

The Knight Cancer Institute at Oregon Health & Science University is supported by a Cancer Center Support Grant from the National Cancer Institute (3P30CA069533). R.C.S. is supported by the National Cancer Institute (R01 grants CA196228 and CA186241), U01 CA224012, and U54 CA209988. G.B.M. received support from the National Cancer Institute P50 CA217685, P50 CA098258, UO1 CA 2197842, SAC11052 from the Susan G. Komen Breast Cancer Foundation, BCRF-19-110 from the Breast Cancer Research Foundation, 545152 from the Ovarian Cancer Research Alliance, and a kind gift from the Sheldon and Miriam Adelson Medical Research

Foundation. J.W.G. received grants from the Susan G Komen Foundation (SAC190012), the Murdock Charitable Trust, the Prospect Creek Foundation, the Brenden-Colson Center for Pancreatic Care, the National Institutes of Health (U54 HG008100) and the National Cancer Institute (U2C CA233280, U54 CA209988, U01 CA195469, U01 CA195469, R44CA224994). J.W.T. received grants from the Mark Foundation for Cancer Research, the Silver Family Foundation, the V Foundation for Cancer Research, the Gabrielle's Angel Foundation for Cancer Research, and the National Cancer Institute (1R01CA183947, 1U01CA217862, 1U54CA224019).

Disclosure Statement

G.B.M. is SAB member or Consultant with AstraZeneca, Chrysallis Biotechnology, ImmunoMET, Ionis, Lilly, PDX Pharmaceuticals, Signalchem Lifesciences, Symphogen, Tarveda, has stock options with Catena Pharmaceuticals, ImmunoMet, SignalChem, Tarveda has licensed technology to Myriad Genetics and Nanostring, and has sponsored research with Nanostring and Ionis. J.W.G. received research support from, Danaher/Cepheid, PDX Pharmaceuticals and Thermo Fisher Scientific (formerly FEI), Zeiss, Quantitative Imaging and Micron. He has licensed technology to Abbott Diagnostics, and he has ownership positions in PDX Pharmaceuticals, KromaTid, and Convergent Genomics. J.W.T. has received research support from Agios, Aptose, Array, AstraZeneca, Constellation, Genentech, Gilead, Incyte, Janssen, Petra, Seattle Genetics, Syros, and Takeda.

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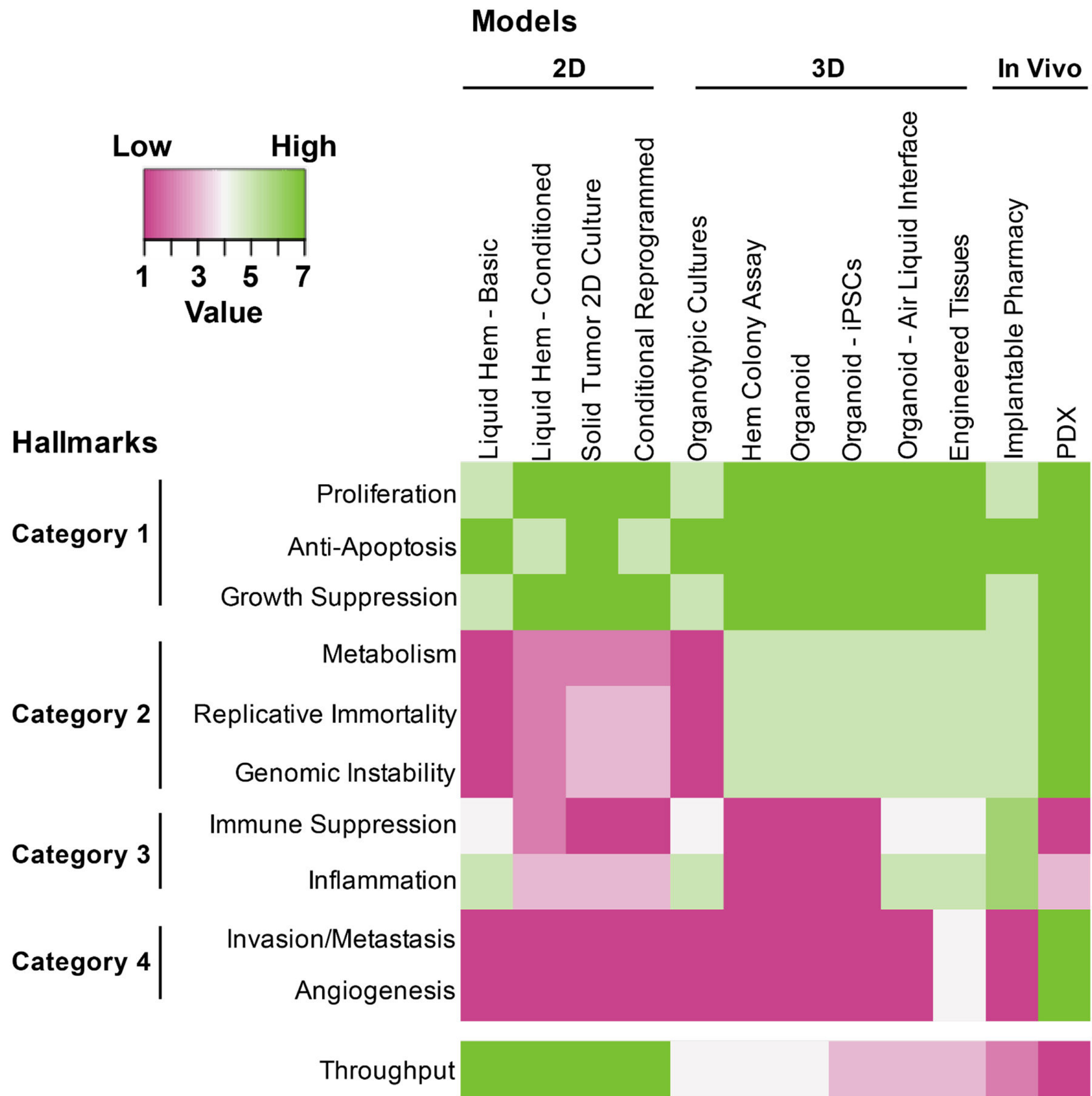

Figure 1.

The 10 hallmarks of cancer are organized into 4 categories based on feasibility of various *ex vivo* platforms to accurately assess sensitivity to drugs targeting those hallmarks. In Category 1, most platforms are highly capable of directly assessing cell proliferation and apoptosis or, at least, assessing biomarkers of these phenotypic states. The Category 2 hallmarks of metabolism, replicative immortality, and genomic instability are better modeled with approaches that enable robust cell proliferation and/or that can readout phenotypes beyond short term cultures. Hallmarks in Category 3 can be best modeled with assays that include heterogeneous cell mixtures that are derived from or mimic the native tumor state. In this way, some of the basic models may actually perform better than models with greater perturbations on the tumor biopsy, however, these heterogeneous cell mixtures may be added back with some of the sophisticated engineered models. Finally, Category 4 hallmarks may be the most challenging, but can be modeled using bioengineered 3-dimensional approaches

or patient-derived xenograft models. In general, the 3-dimensional and *in vivo* approaches are able to accurately model a larger number of hallmarks, though it is worth noting that these strategies currently do not have the throughput of the more basic approaches.

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