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Consideration of the cellular microenvironment: Physiologically relevant co-culture systems in drug discovery☆

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ABSTRACT

There is renewed interest in phenotypic approaches to drug discovery, using cell-based assays to select new drugs, with the goal of improving pharmaceutical success. Assays that are more predictive of human biology can help researchers achieve this goal. Primary cells are more physiologically relevant to human biology and advances are being made in methods to expand the available cell types and improve the potential clinical translation of these assays through the use of co-cultures or three-dimensional (3D) technologies. Of particular interest are assays that may be suitable for industrial scale drug discovery. Here we review the use of primary human cells and co-cultures in drug discovery and describe the characteristics of co-culture models for inflammation biology (BioMAP systems), neo-vascularization and tumor microenvironments. Finally we briefly describe technical trends that may enable and impact the development of physiologically relevant co-culture assays in the near future.

Contents

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1. Introduction

Over the past 30 years, robotics and high throughput screening (HTS) technologies have revolutionized the drug discovery process, influencing cell-based as well as biochemical approaches. Cell-based assays enable selection and characterization of compounds based on functional effects in intact cells. These functional effects can be measured in a variety of ways including: changes in cell components (e.g. protein, mRNA, or metabolite levels) or component states (phosphorylation state, methylation, etc.), physical properties of cells (e.g. shape, proliferation, chemotaxis or impedance), or in the subcellular localization of organelles or molecules (e.g. as can be assessed by high content screening). The advantages of cell-based assays over biochemical assays include the ability to (1) assess targets in physiologically relevant settings, (2) evaluate entire pathways and multiple targets in a single assay format, and (3) characterize compounds with unknown targets or targets that are not amenable to biochemical approaches. Cell-based assays can range in their suitability for high throughput compound testing, however, and in the past, the more physiologically relevant but complex assays have been restricted for use in the evaluation of small numbers of test agents. As interest in phenotypic drug discovery increases, so does interest in methods for developing physiologically relevant assays that are also suitable for industrial drug discovery.

In the pharmaceutical industry most cell-based screening is performed in immortalized cell lines, often engineered to overexpress targets or reporter constructs. Cell lines are attractive to use, due to their ease of culture, expansion potential, and suitability for the prosecution of high-throughput screens. However, generation of cell lines involves the identification of cell clones which differ from their in vivo counterparts by proliferating robustly ex vivo, an experimental condition which may select for cell clones exhibiting enhanced growth characteristics and potentially altered regulatory and signal transduction pathways. Since correlation of in vitro and in vivo studies are frequently discordant, efforts to develop more physiologically relevant in vitro assays which better translate to in vivo biology are of fundamental importance.

For assays to be used for phenotypic drug discovery, in programs without prior identification of a molecular target, it is important to establish that the assays to be used are validated to be relevant to the disease process. Although technically challenging, primary human cells are attractive to use for screening as they are phenotypically more similar to normal cells and retain the normal regulation of their growth pathways. Primary human cells are now available from many tissues [1]. And while three dimensional (3D) model systems and engineered tissues are attractive to pursue as they can provide architecture that is considered more physiologically relevant, due to their limited scalability, they are currently more applicable to basic research or transplantation medicine rather than industrial drug discovery.

We have found that the use of primary human cell-based co-cultures provides a significant step towards physiological relevance, but in two-dimensional (2D) formats that are more easily scaled. Here we will focus on the use of primary human cells and co-cultures in industrial drug discovery applications, as their utilization is becoming more widespread.

2. Industrial drug discovery

The pharmaceutical drug discovery process has a number of steps that can be subdivided into preclinical and clinical components (Fig. 1A). In the pre-clinical or discovery phase, pharmacologically active agents are identified and optimized in the lead generation and optimization phases, respectfully. For small molecules, compounds are screened in medium throughput (MTS) or in high throughput (HTS), corresponding to tens of thousands or hundreds of thousands of molecules, respectively. Following initial testing, compound activity is confirmed in dose response experiments and the structures of active molecules of interest are used to identify similar or related untested molecules, a process called hit expansion. Compounds with a combination of promising potency, efficacy, chemical structure, and physical properties and that demonstrate structure activity relationships are tested in pre-clinical disease models and used as the template for subsequent cycles of chemical synthesis and pre-clinical testing in animal models. Identification of a safe and therapeutically efficacious compound in animal models allows selection of a clinical candidate which is scaled up for clinical trial safety testing in healthy human volunteers or patients (Phase 1), dose finding testing in patients (Phase 2) and final efficacy testing in patients (Phase 3). It should be noted that the attrition rates of preclinical, Phase 1, Phase 2, and Phase 3 have been estimated as approximately 80%, 50%, 70%, and 50%, respectively [2] which underlines the many intrinsic hurdles and high risk nature of drug discovery. Indeed, it has been this high rate of failures in clinical testing that drives the interest in pursuing more physiologically relevant screens in drug discovery.

The topic of this review, physiologically relevant co-culture assay systems, is an important component of the discovery, and preclinical phases of the drug discovery process as illustrated in greater detail in Fig. 1B. The goal of preclinical research is to identify a molecule that is safe and efficacious in animal models and that is also likely to be active in humans, where human cellular models of therapeutically relevant conditions can be applied. Although conceptually straightforward, the majority of clinical trials fail due to lack of human efficacy [3]. This illustrates practical difficulties of translating preclinical results to clinical trials and simultaneously underlines the scientific and business drivers for development of preclinical models with enhanced clinical translation such as disease relevant in vitro assay systems.

Contemporary drug discovery research has relied heavily on the identification of a molecular target thought to be physiologically relevant and where in vivo modulation of activity is expected to be therapeutically beneficial. Such hypothesis driven drug discovery approaches have been termed “targeted drug discovery” (TDD) and have been popular since the integration of molecular biology capabilities and the elucidation of novel drug targets from exon and genomic sequencing [4]. Typically in TDD, drug target specific assays are enabled and utilized for MTS or HTS and target selectivity assays frequently follow to establish the specificity of confirmed actives to the molecular target of interest. If biochemical screening/selectivity assays were utilized for screening, cell based assays (frequently using genetically engineered cell lines overexpressing the molecular target and/or substrate) are utilized to determine whether confirmed biochemical actives modulate the molecular target in a cellular context (Fig. 1B). In the TDD strategy the ability of a compound to modulate a therapeutically relevant biomarker or response in a physiologically relevant cellular system is not addressed until several steps beyond MTS/HTS and just preceding in vivo testing (Fig. 1B).

Interestingly, despite the emphasis on hypothesis driven TDD approaches, phenotypic drug discovery (PDD) approaches account for the majority of first-in-class new molecular entities (NMEs) that have attained US FDA approval [5,6]. Unlike TDD, the PDD strategy is empirical and relies on direct chemical interrogation of a physiologically relevant biological system to identify compounds that modulate therapeutically relevant endpoints [4].

Physiologically/therapeutically relevant cellular systems can therefore be positioned at various stages of the preclinical discovery workflow depending on the specific needs of the project and the choice of lead generation strategy (Fig. 1B) which in turn defines the prerequisite operational parameters, throughput, and level of statistical validation required from the assay. For example projects utilizing a PDD strategy may utilize co-culture systems in lead generation and compound screening where tens to hundreds of thousands of compounds are initially tested whereas TDD approaches may utilize the same physiologically relevant cellular model two or three steps following lead generation and may be required to test hundreds of compounds. Co-

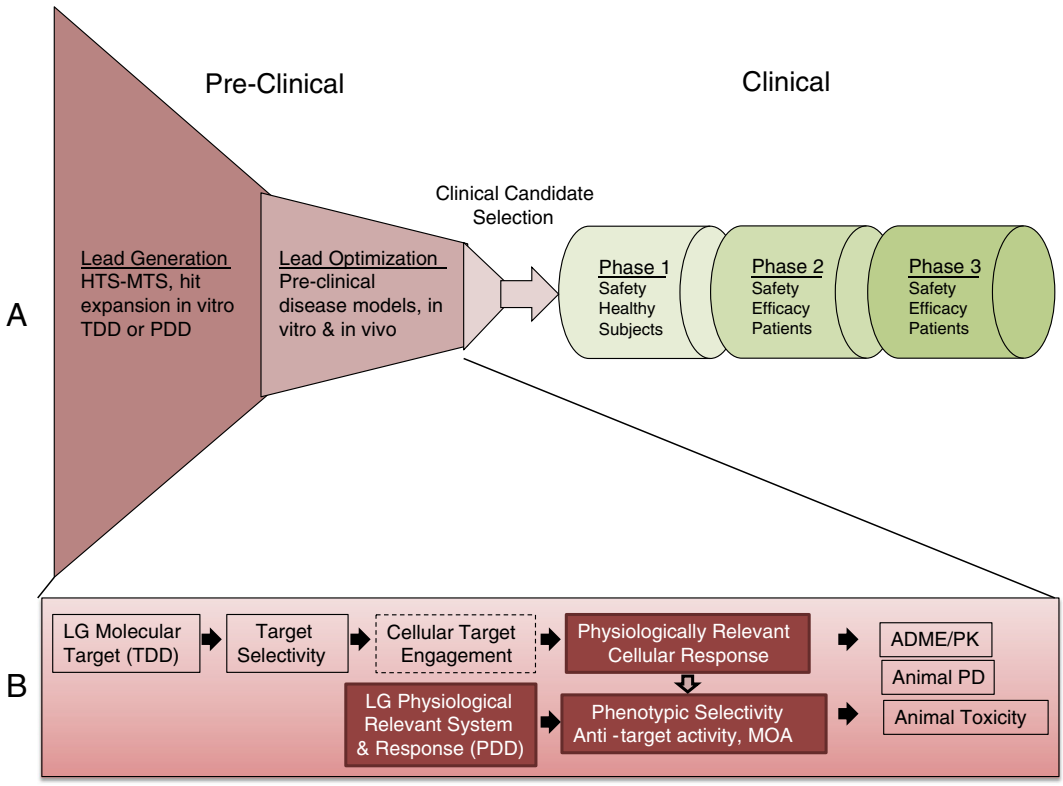

Fig. 1. The drug discovery process is composed of pre-clinical and clinical components (A). In the pre-clinical or discovery phase, pharmacologically active agents are identified and optimized in the lead generation and optimization phases, respectfully. Molecules that fulfill in vitro success criteria and are safe and efficacious in animal disease models can be advanced to a clinical candidate selection. Clinical trials are conducted in three phases, safety testing in healthy human volunteers or patients (Phase 1), dose finding testing in patients (Phase 2) and final efficacy testing in patients (Phase 3). The role of physiologically relevant cell assays are differentially positioned within pre-clinical project flow schemes depending on the lead generation strategy utilized (B; highlighted). The pharmaceutical industry currently utilize hypothesis driven, molecular target centric approaches for lead generation in the majority of cases. This targeted drug discovery (TDD) relies on the causal linkage of a molecular target with the disease condition (target validation) for success. In contrast, most first in class drugs were initially identified by approaches that directly interrogated the physiologically relevant biology (2, 3) in a molecular target agnostic manner. Such empirical lead generation approaches have been termed phenotypic drug discovery (PDD) and utilizes physiologically relevant systems in the initial lead generation stage whereas TDD typically utilizes these cellular systems several steps following compound screening (B).

culture assays may be used to investigate phenotypic selectivity, mechanism of action, and anti-target selectivity that may require testing between tens of compounds for TDD or several hundred compounds for PDD (Fig. 1B). In addition to assay capacity the specific purpose of a co-culture assay will in part determine the modality in which the assay is operated; lead generation/screening will generally require single point determinations initially whereas assays deeper into the project flow scheme will typically be done in dose response. Assay capacity and modality subsequently define the level and type of statistical validation that is relevant (summarized in the Lilly-NIH Assay Guidance Manual; http://www.ncbi.nlm.nih.gov/books/NBK83782/). This is further discussed in Section 2.2 below.

2.1. Cell-based assays in drug discovery

Cell-based assays are not without certain limitations. They are technically more challenging with a greater number of methodological steps and experimental variables. Culture media typically contain hundreds of components and incorporate various base media differing in buffering capacity and pH; moreover additional factors such as tissue culture plate composition and surface coating can influence cell growth and function in complex, often poorly understood ways. In addition, as cells are cultured and passaged, the ability of cells to respond to many environmental cues creates phenotype drift over time, making it challenging to develop assays that are sufficiently reproducible [7]. Phenotypic variation can also lead to culture artifacts, and altered cell behaviors that are not representative of cell function in vivo and that lead to misleading conclusions. The combinatorial complexity of cellular assays includes the cell type (established lines, primary cells, stem cell derived cells, co-cultures/mono culture), species, and cell culture media/matrix conditions. Therefore the variation of signal transduction states, pharmacology and cell health/proliferation can be very large. This makes it difficult to define a priori the best optimal conditions, although there are best practices to achieve such conditions (see below). Such details are unfortunately system specific and must be determined in the context of the specific cellular assay including the experimental objective, assay capacity, statistical validation requirements, operational details/duration, and availability/reproducibility of cells and components. For development of cell-based assays one needs to make trade-off decisions balancing practical considerations such as availability, scalability, and operational robustness with degree of "physiological relevance" needed for the functional study and previous information and/or likelihood of in vitro–in vivo correspondence [8]. Finally, it is important to note that although appropriate, non-recombinant cell-based assays are likely to better mimic in vivo biology and identify active compounds that are more likely to be active in vivo, as compared to corresponding actives from biochemical screens, cell-based assays are still a long way from the intact organism, missing cues from other cells, tissues and organ systems, and typically insensitive to compound features that determine absorption, metabolism, distribution and excretion. Table 1 lists advantages and disadvantages of cell types and assay formats for screening.

Table 1
Cell types and assay formats: advantages and disadvantages.

Cell types	Advantages	Disadvantages
Cell lines	Availability of large cell numbers from a single genetic donor	Potential for abnormal regulatory mechanisms
	Highly suitable for miniaturization (1536-well)	Phenotypic drift in culture
Primary cells	Physiologically relevant	Not all cell types are available
	Endogenous pathways	Cell numbers (per donor) are limited
		Management of donor to donor variation is required
		Expensive
		Phenotypic drift in culture
Stem cell-derived cells	Potentially physiologically relevant	Uncertain reproducibility in differentiation and physiological response
	Access to rare cell types	Cell numbers (per batch) are limited
	Scale up of cells from single donors is possible	Expensive
		Phenotypic drift in culture
Format	**Advantages**	**Disadvantages**
Monoculture	Convenient	Less physiologically relevant than co-culture or 3D
	Many published methods	Higher requirement for ECM, exogenous factors
Co-culture	More physiologically relevant than monoculture	Experimental set up is more involved
	Fewer numbers of cells required than monoculture	Selection of assay media can be complicated
	Cell–cell cross talk is present	Few published methods
3D	More physiologically relevant than 2D	Lower throughput than 2D assays
	Tissue architecture is present	Responses are variable through the 3D structure
	Many approaches available (scaffolds, hanging drop, etc.)	Physiological relevance of various methods uncertain
		Experimental endpoints are limited
3D co-culture	Most physiologically relevant systems	Technically most involved
	Tissue architecture is present	Lower throughput than 2D assays
	Many approaches available (scaffolds, hanging drop, etc.)	Physiological relevance of various methods uncertain
		Experimental endpoints are limited

2.1.1. Cell type selection

As stated previously assay and cell culture conditions should be as physiological as practical or as required for the application at hand. Physiological relevance is of particular importance for cell-based assays used in phenotypic drug discovery (PDD) programs. Phenotypic drug discovery uses functional assays, either cell-based or in vivo disease models, for identifying potential drugs in a molecular target agnostic manner. In some instances non-recombinant human or non-human cell lines are the only practical solution but are non-ideal in regard to their origin, immortalized state or potential for dysregulated signaling mechanisms. Primary human cells are attractive to consider since they are not immortalized and without the dysregulated signaling mechanisms that characterize cell lines. While early passage primary cells have had less of an opportunity to lose these physiologically relevant regulatory pathways, this is not always the case. Primary hepatocytes rapidly lose glucagon signaling upon cell culture (JAL, not shown), and high endothelial venules lose their expression of the lymphocyte homing receptor ligands, the mucosal and peripheral lymph node addressins (ELB, data not shown).

The development of reproducible assays using primary cells that are amenable to automation and high-throughput screening remains challenging. Although a wide variety of primary cell types and culture methods for these cells have become available over the last several decades, the number of cells that can be obtained from a single donor is limited and procedures for expanding cell numbers in culture are not available for every cell type or are limited to a few passages. For large screens, the number of assays that need to be run often requires that the work be performed in batches, sometimes over a period of months or years [9,10]. For this reason, large screens using primary cell assays often require using cells from multiple donors. The need to accommodate donor-to-donor variation contributes to the challenge of developing reproducible assays. It is challenging to develop primary cell-based assays that retain these regulatory mechanisms but yet are reproducible and amenable to automation and high-throughput screening. This situation often requires management by prescreening donors, pooling cells from multiple donors, and using data normalization methods to reduce donor variation.

In the past, isolation and culture of primary human cells required access to human tissues as well as having to make media from individual components, many of which were of uncertain quality (e.g. bovine brain extract) [11]. Over the years, methods for the isolation and culture of a diversity of primary human cell types have been developed and now many cell types and specialized media are available from commercial vendors. Available primary cell types include blood and immune cell types, stem cells, endothelial cells, epithelial cells, fibroblasts, adipocytes, hepatocytes, muscle cells, astrocytes and many others [1,12–17].

Blood cells and blood cell-derived cell types are readily available and many examples exist where they have been used for high throughput screening [18]. In an image-based screening campaign, human CD34+ mesenchymal stem cells were used to screen a 50,000 compound library for regulators of megakaryopoiesis and resulted in the identification of PDGFR inhibitors as promoters of megakaryocyte differentiation [19]. Novel SYK kinase inhibitors were identified in a screen using cultured human mast cells, and eventually led to the discovery of fostamatinib, currently in clinical testing for idiopathic thrombocytopenic purpura, IPT [20].

Methods for primary human cell culture continue to improve, in some cases with the assistance of high throughput screening efforts. Hepatocytes have been of intense interest to pharmaceutical scientists in metabolism and toxicology, although they have been challenging to culture [17]. Shan and co-workers [21] performed a screen in primary human hepatocytes and identified compounds that promote hepatocyte proliferation and other compounds that promoted the differentiation of induced pluripotent stem cell (iPS)-derived hepatocytes. In this way, high throughput technologies developed over the past several decades are helping to advance the science of primary human cell culture.

Stem cell approaches are a growing area of research and are providing access to primary cell-derived cell types that have been more difficult to access or culture, such as neurons, oligodendrocytes, intestinal epithelial cells and pancreatic beta cells. Stem cells include embryonic stem cells, mesenchymal stem cells and induced pluripotent stem cells (iPS). Assays using human adipose-derived stem cells to study lipogenesis have been described [22]. iPS technologies also provide the potential for patient-tailored assays, and the ability to generate large

numbers of cells with a specific genetic background (e.g. mutations) [23]. iPS derived astrocytes from Huntington's patients have been developed that replicate the vacuolar phenotype of cells found in patient tissues [24,25]. Peng and co-workers (2013) screened a set of Parkinson's disease drug candidates in iPS-derived dopaminergic neurons [26]. While stem cell technologies offer the opportunity to access new cell types, potential issues such as the reproducibility of the differentiation process and the unknown degree in which stem cell derived cell types mimic primary cells and in vivo biology should be considered.

The historical importance of primary cell mono-culture coupled with advances in media and cell culture methodologies has facilitated the use of primary cell assays in industrial drug discovery [18,27]. The development and refinement of technologies to develop iPS cells coupled with improved methods to reproducibly, differentiate iPS cells into phenotypically distinct cell types exhibiting characteristics of endothelial, neuronal, cardiomyocyte, and hepatocytes foreshadow the integration of iPS derived cell types into drug discovery flow schemes [28], the development and use of patient derived iPS derived cell lines [23,25], and inevitable integration of these primary and iPS derived mono-culture cells into co-culture systems. Considerations for assays suitable for industrial drug discovery are listed in Table 2.

3. Primary cell co-culture assays in industrial drug discovery

Cell–cell interactions and the cell–matrix interactions that are characteristic of tissues help cells retain their normal physiology. This has led to interest in the development of co-culture models, both 2D and 3D for their greater physiologic relevance. Co-cultures have had a long history in basic research for studying cellular and drug mechanisms of action, for example the mixed lymphocyte reaction and T cell assays in immune biology [15,16], and for developing engineered tissues [29–31]. Only more recently, however, have primary human cell co-cultures begun to be applied in industrial settings for high-throughput drug screening.

3.1. Technical considerations for primary human co-culture assays

In contrast to basic research, industrial drug discovery requires that assays provide consistent and quantitative data for efficient compound screening, medicinal chemistry structure activity support, and lead optimization. In order to develop quantitative and highly reproducible assays for drug discovery flow scheme support, assays need to be robust, statistically validated and utilize components that are readily available and of consistent quality for the projected lifetime of the project or assay panel, frequently multiple years. Measures of assay performance include the ratio of signal to background (S/B), coefficient of variation (%CV, the ratio of standard deviation to the mean as a percentage), and Z' factor, a measure of statistical effect size which incorporates both elements (http://www.ncbi.nlm.nih.gov/books/NBK83782/). In this section we provide an overview of the few published examples of human co-culture systems that operate as processes to support industrial drug discovery.

There are numerous technical considerations for developing primary human cell-based co-culture assays. These include the cell types to be combined, the timing of addition of each cell type and length of the culture, the relative numbers of cells, the use or selection of extracellular matrix coating and media formulations. Culture media contains hundreds of compounds and cell types that differ in their preference for components such as glucose, insulin, transferrin, hydrocortisone, serum, amino acids, glucose vitamins, iron, among other components, as well as base media which differ in salts used for buffering, osmolarity, and pH. Brunner et al., 2010 have recently provided an update of various culture media formulations including serum free media [32]. Determining suitable media often requires extensive empirical testing.

The following sections detail primary cell and co-culture models developed in our laboratories for the eventual goal of high-throughput drug screening applications. The first section describes an assay panel comprised of multiple primary human cell types and co-cultures that has been utilized for drug and compound characterization for a number of years. In the second section, we describe the development of a co-culture assay for neovascularization, that as a 2D system, provides for automated imaging and enabled screening of a 32,000 compound collection. The third section describes efforts to study the tumor microenvironment and the role of cancer associated fibroblasts using co-culture models. This work emphasizes the bidirectionality of effects that can be captured: of tumor cells on host cells and host cells on tumor cells.

3.2. An assay panel utilizing human primary cell based co-cultures

We have previously described a phenotypic assay platform that uses a suite of primary human cell-based assays, BioMAP systems, for the characterization and functional classification of chemicals and bioactive

Table 2
Assay selection considerations.

Feature	Options	Factors to consider
Biology of interest	Novel or validated biology	If biology is novel, there may be few tools available and higher project risk. If there are known pathways and targets already identified for the biology of interest, this will guide the selection of follow up and secondary assays. The level of validation for disease relevance (in vitro, in vivo, animal versus human, genetic association, clinical biomarker, etc.) can mitigate project risk.
Selection of cell type	Cell line, primary cell, stem cell, iPS cell, stem-cell derived cells	Availability of cells, culture methods, expansion and cell banking as well as cost are factors. Primary cells and iPS-derived cells are more physiologically relevant, but also more costly.
Number of donors	Single or pooled donors	Are there genetic contributions that must be considered? If so, methods that allow screening of single donors must be used (i.e. cell lines or stem-cell derived cells). Assay scale is also a factor for primary cells in determining the number of donors that will be required to complete a screen.
Assay scale	Low (<1000), medium (1000–30,000), or high throughput (>100,000)	How many compounds are to be screened? Can the compounds be pre-screened or filtered prior to screening? Can the assay be miniaturized?
Time course	Minutes, hours, days	What is the time course of the assay? Minutes, versus hours, versus days? Very short assays can be logistically problematic at high scale due to robotics programming, etc. Very long assays are subject to risk of evaporation and contamination.
Assay characterization	Positive and negative controls, pathway inhibitors, siRNA library, etc.	How well has the assay been characterized phenotypically? With cell-type-specific biomarkers or by responses to positive and negative controls? Interrogation by inhibitors of common pathway mechanisms or siRNA knock down methods is highly recommended.
Statistical performance	Signal to noise, Z' factor, experiment-to-experiment variability should be established.	Z' > 0.5 are preferred.
Flow scheme for triaging active compounds	Other cell lines, other cell types, other pathway assays, in vivo studies	How will active compounds be further characterized? If the endpoint is general (cell health or proliferation), follow up assays will need to be specific (e.g. assay cell type versus other cell types).
Endpoint selection and technology	Protein, mRNA, metabolite, ATP, morphology, etc.	Homogeneous assays are the most convenient for high throughput assays. Often there is a tradeoff between statistical performance and biological relevance.

agents [10,33–35]. Each assay system contains primary cells or co-cultures in stimulatory conditions that reflect various aspects of tissue and disease biology of different tissue types: the vasculature, skin, lung, immune and inflammatory tissues. A key aspect of these systems is the use of combinations of stimulatory factors and cell types to mimic the in vivo pathophysiological state of the affected tissues. Because of the networked architecture of signal transduction systems within cells, these assays produce cell responses that are qualitatively and quantitatively different than typical monoculture assays, and are more representative of physiologic settings and disease states where multiple factors and cell types are present [33].

In each of these assay systems, the levels of a selected set (8–12) of translational biomarkers, proteins and metabolites, are measured. The use of these biomarkers as endpoints has the advantage of providing information that can be used directly for translational studies in animals and humans. Endpoints include disease biomarkers (cell surface and secreted receptors, ligands, enzymes, matrix components), molecules involved in cell–cell interactions or cellular communication, mediators or regulators of the host responses (e.g. inflammatory, coagulation and fibrinolytic responses) and risk factors for particular disease outcomes [10,33].

3.2.1. Performance of primary human co-cultures versus monocultures

The three BioMAP systems, shown in Table 3, illustrate some of the features and performance characteristics of these assays. All three of these systems model vascular inflammation, either in a monoculture (3C system) or co-culture (SAg and LPS) format. The 3C system contains primary human vascular endothelial cells (HUVEC) stimulated with a combination of cytokines (IL-1beta, TNF-alpha and IFN-gamma), whereas the LPS and SAg systems are co-cultures of HUVEC and peripheral blood mononuclear cells (PBMC), stimulated with lipopolysaccharide to activate monocytes through the toll-like receptor 4 (TLR4) or with superantigens to activate T cells through the T cell receptor (LPS and SAg systems respectively). In these assays, the levels of a set of biomarkers (listed on the x-axis in Fig. 1) are measured 24 h following addition of stimuli to the cultures in the presence or absence of test drug.

One of the biomarkers measured in all three systems is the endothelial cell adhesion molecule E-selectin that mediates adhesion and recruitment of circulating leukocytes and skin-homing memory T cells into sites of inflammation, and has been shown to be a clinical biomarker for infection, diabetes, obesity and the systemic inflammatory response syndrome [36–40]. The measurement of E-selectin in all three of these systems allows comparative analysis of the regulation of E-selectin that illustrates features of co-cultures versus monocultures under distinct physiological contexts. E-selectin is increased in endothelial cells following the activation of cytokine, T cell, and Toll receptors in these systems, however, as shown in Fig. 2 and Table 4, it is distinctly regulated in different physiological contexts. Fig. 2 shows that E-selectin levels are reduced by BIRB-796, a p38 MAPK inhibitor, in the LPS system, but not in the 3C or SAg systems. Table 4 provides an expanded list of mechanisms that have been shown to regulate E-selectin levels in each of these systems. As shown in this table, E-selectin is responsive to different mechanisms of action in the co-culture systems (LPS and SAg systems) than in the monoculture system (3C). Remarkably, 17 of 28 different target mechanisms evaluated were shown to regulate E-selectin in the SAg system compared to 6 each in the LPS and 3C systems.

Statistical robustness and reproducibility are foundational for assays used in industrial drug discovery. Fig. 3 shows the relative expression and performance metrics of E-selectin in these assays. E-selectin is induced to similar levels in each of these systems (Fig. 3A). Notably the average coefficient of variation across >290 assays performed over 5 years is well below 10% for all three assay types (Fig. 3B), and demonstrates that the performance of a co-culture assay (LPS assay) can be as good as a monoculture assay (3C system).

The regulation of E-selectin by many pathway mechanisms is not unusual, but rather, is a general feature of phenotypic assays. Thus, when phenotypic assays are used for primary screening, it is important for the drug discovery flow scheme to include methods to rapidly classify the resulting hits into mechanistic classes, so that known target mechanisms can be quickly identified. This illustrates the importance of counter-screening strategies to triage phenotypic screening hits early in PDD programs, and also the need for primary screens to be well characterized for possible known mechanisms prior to running a large screening campaign.

3.2.2. Predictive models for mechanism classes from co-culture assay data

One of the interesting applications of the BioMAP platform has been to employ a panel of assay systems, together with a large compound reference data set, to build predictive models for mechanism classes based on profile signatures. BioMAP assays have been automated and applied to build a profile database of >1000 bioactive agents (drugs, biologics, experimental and environmental chemicals) that includes data generated from 100s of experiments performed over a period of years. BioMAP signatures have been shown to distinguish compounds based on mechanism of action (MoA) and target selectivity, and have been correlated to in vivo biology [10,33,34]. This approach has been useful for the selection and characterization of drug leads for diverse target classes (including kinase, nuclear hormone receptor) and to understand compounds with unknown MoAs [34,41–43]. This method can be applied to triage phenotypic drug discovery hits, as described by Berg, 2013 [44]. Interestingly, evaluation of various screening collections reveals that the most frequent hits from PDD screens include mitochondrial and microtubule inhibitors, cAMP elevators and epigenetic modulators.

Toxicity associated mechanisms that have been detected and classified by this approach include inducers of endoplasmic reticulum stress, mitochondrial dysfunction, immunosuppression, and microtubule inhibition [10,44,45]. This method may be useful for predictive toxicity applications in cases where targets are not amenable to biochemical screening (e.g. mitochondrial targets) or are unknown.

As with all cell-based approaches, there are limitations. First, not all targets will be present in the panel of assay systems tested, or will be functionally detectable. The readout measurements, many of which are known clinical or disease biomarkers, are downstream of multiple pathways. In addition, as mentioned above, reagents or methods for detection of desired biomarkers or assay endpoints may not be available. Finally, there are aspects of in vivo biology that may never be incorporated into 2D co-culture models but will require more sophisticated approaches involving 3D methods and/or microfluidics.

3.3. Neo-vascularization as a drug discovery assay

Modeling the process of blood vessel formation, or neovascularization is typically thought to require 3D methods. However, recent results suggest that neovascularization models that are 2D, which can be more easily performed in high-throughput, may be also useful. Neovascularization is a complex process which requires the migration, differentiation, physical interaction, and coordinated cellular signaling of precursor and mature forms of endothelial cells with stromal cells (fibroblasts, smooth muscle cells, and pericytes) coupled with morphological changes including endothelial tube formation, anastomosis, endothelial sprouting, and endothelial vessel stabilization by pericyte recruitment/differentiation. While vascular endothelial growth factor, VEGF is recognized as the dominant pro-angiogenic factor, other factors such as fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, angiopoietins, thrombomodulin, notch ligands and transforming growth factor-b are involved as well (see [46,47] for reviews).

It is difficult to simulate a complex biology like angiogenesis using immortalized cell lines. As an alternative, Kubota et al. [48] observed

Table 3
Selected BioMAP systems. Assays and method have been described previously [10].

System	Cell types/stimuli	Biomarker endpoints	System description
	Venular endothelial cells/IL-1β + TNFα + IFN-γ	VCAM-1, ICAM-1, uPAR, Eselectin, MCP-1, MIG, HLADR, IL-8, TM, TF, Proliferation, SRB, Vis (13)	The 3C system consists of primary human umbilical vein endothelial cells stimulated with IL1β + TNFα + IFNγ for 24 h. These conditions model vascular inflammation of the Th1 type, an environment that promotes monocyte and T cell adhesion and recruitment and is anti-angiogenic. This system is relevant for chronic inflammatory diseases, cardiovascular disease and restenosis
	Peripheral blood mononuclear cells + endothelial cells/TLR4	CD40, VCAM-1, Tissue Factor, MCP-1, E-selectin, IL-1β, IL-8, M-CSF, TNF-α, PGE2, SRB (11)	The LPS system consists of primary human umbilical vein endothelial cells cocultured with peripheral blood mononuclear cells and stimulated with very low amounts of bacterial endotoxin (ligand for toll-like receptor 4, TLR4). These conditions model chronic inflammation of the Th1 type and monocyte activation responses. This system is relevant to inflammatory conditions where monocytes play a key role including atherosclerosis, restenosis, rheumatoid arthritis, and other chronic inflammatory conditions, as well as metabolic diseases.
	Peripheral blood mononuclear cells + endothelial cells/TCR	MCP-1, CD38, CD40, Eselectin, CD69, IL-8, MIG, PBMC Cytotoxicity, Proliferation, SRB (10)	The SAg system consists of primary human umbilical vein endothelial cells cocultured with peripheral blood mononuclear cells and stimulated with a cocktail of superantigens (ligands for the T cell receptor). These conditions model chronic inflammation of the Th1 type and T cell effector responses to TCR signaling with costimulation. This system is relevant to inflammatory conditions where T cells play a key role including organ transplantation, rheumatoid arthritis, psoriasis, Crohn's disease and multiple sclerosis.

that primary endothelial cells cultured on matrigel, a basement membrane matrix extract containing endogenous growth factors, form structures which morphologically resemble capillaries. This assay has been foundational in the angiogenesis field for over 25 years (see [49] for review) but has limited physiological relevance as it captures exclusively endothelial events and not the contribution of the various stromal components, endothelial–stromal interaction, or diverse other factors that modulate neo-vascularization.

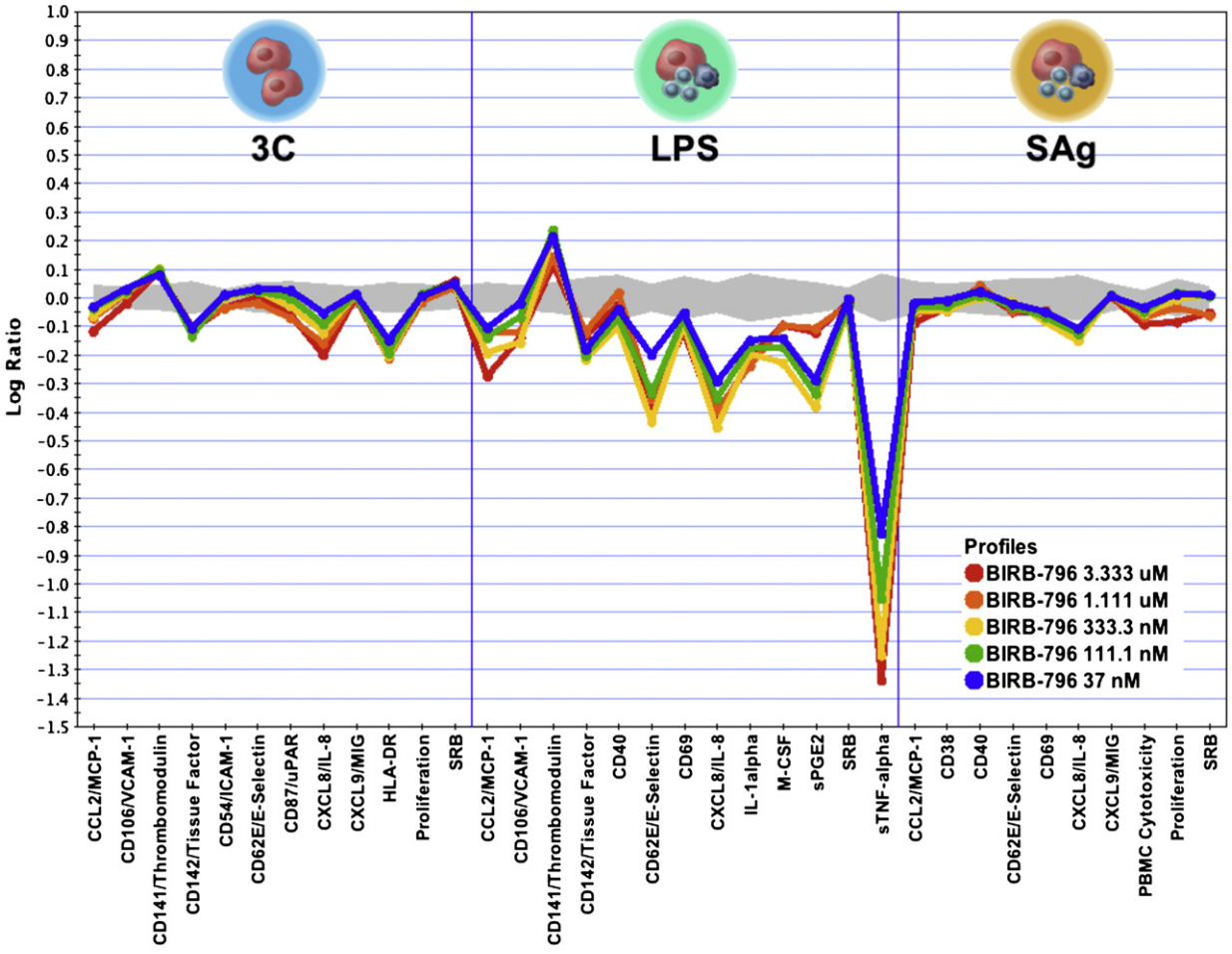

Fig. 2. BioMAP profile of BIRB-796, a well-characterized p38 MAP kinase inhibitor. BIRB-796 was tested in 3 BioMAP model systems at 3.33 microM (red), 1.11 microM (orange), 333 nM (yellow), 111 nM (green), and 37 nM (blue), as previously described [10]. The protein biomarkers measured are indicated along the x-axis. The y-axis shows the log expression ratios (log10 [parameter value with drug/parameter value of control]) of the readout protein levels relative to solvent controls. The shaded gray region around the y-axis origin indicates the 95% significance envelope of control samples (no drug). Note the reduction of E-selectin in the LPS system, but not in the 3C or SAg systems.

Table 4
Pathways that regulate E-selectin in monoculture (3C) versus co-culture (LPS and SAg) BioMAP systems. Twenty-eight pathways were assessed by chemical biology perturbation with a panel of well-characterized tool compounds [44]. Compounds with the pathway mechanisms listed either reduce the levels of E-selectin in the indicated system (red), increase the levels (green) or have no effect (black).

Mechanism Class	3C	LPS	SAg
20S Proteasome Inhibitor			
AhR Agonist			
Calcineurin Inhibitor			
EGFR Inhibitor			
EP Agonist			
ER Agonist			
GR Agonist (Full)			
H1 Antagonist			
HDAC Inhibitor			
HMG-CoA Reductase Inhibitor			
Hsp90 Inhibitor			
IKK2 Inhibitor			
IL-17A Agonist			
JAK Inhibitor			
MEK Inhibitor			
Microtubule Disruptor			
Microtubule Stabilizer			
Mitochondrial Inhibitor			
mTOR Inhibitor			
p38 MAPK Inhibitor			
PDE IV Inhibitor			
PI3K Inhibitor			
PKC (c+n) Inhibitor			
RAR/RXR Agonist			
SR Ca++ ATPase Inhibitor			
Src Family Inhibitor			
TNF-alpha Antagonist			
Vitamin D Receptor Agonist			

3.3.1. Co-culture models for neovascularization

In order to include the stromal cell component, primary fibroblasts were initially co-cultured with terminally differentiated primary endothelial cells [50]. Quantitative image analysis of endothelial tubules formed on matrigel versus fibroblast co-cultures indicate that the formation of morphologically distinct networks in the endothelial-network formed by co-culture appears more representative of capillary formation in vivo [51]. Endothelial tube formation in the fibroblast co-culture assay is dependent on exogenous angiogenic factors and is stimulated by VEGF, bFGF, and EGF [52] with maximal endothelial tube formation observed between 10 and 14 days [50,52]. Formation of endothelial cords are prevented by VEGFR and FGFR biased receptor tyrosine kinase inhibitors, axitinib and PD-161570 respectively, in a dose dependent manner [52]. Isherwood et al. has utilized this co-culture system to enable an automated, high content assay platform where multi-parametric fingerprints can be utilized to classify the phenotypes resulting from treatment with compounds modulating diverse molecular mechanisms [53].

Other in vitro models take into account the fact that blood vessels are composed of three cell layers. The intima, the inner layer, is a monolayer of endothelial cells; the media is composed of smooth muscle-like cells and extracellular matrix in mature vessels; the adventitia, the outer layer, contains extracellular matrix, fibroblasts, capillaries and nerves. Functional interactions between EC and SMC have been studied by direct co-culture followed by separation of the cell types with magnetic beads [54]. SMC in co-cultures exhibited increased expression and protein levels of PDGF and TGFb, increased expression of VEGF, and decreased expression of bFGF and bFGFR relative to mono-cultured SMC. EC in co-cultures exhibited higher expression and protein levels of VEGF, but unaltered expression of PDGF and TGF-b relative to mono-culture EC. This study underlines the importance of direct cell interaction between EC and SMC to regulate the expression and secretion of factors necessary for angiogenesis and vessel maturation [55].

Evensen et al. utilized time lapse live cell and immuno-fluorescence microscopy to study the cellular phenotypes and tube formation ability of GFP-labeled EC in direct co-culture with vSMC [56]. Addition of GFP-EC to a monolayer of vSMC inhibited EC proliferation and led to Rac1 dependent formation of a polarized, migratory EC morphology which resulted in the formation of stable endothelial networks. Immunofluorescence of the resulting networks indicated the formation of adherens junctions containing VE-cadherin, a structure typical of homotypic EC–EC interactions, and the biosynthesis of a collagen IV region juxtaposed with the EC network consistent with the deposition of a basement membrane structure. Taken together this study indicates that EC-vSMC direct co-culture leads to altered cellular behaviors, morphology changes, and biosynthetic functions that reflects several facets of blood vessel formation [56].

Following formation of the initial endothelial plexus, recruitment/differentiation of smooth muscle-actin (SMA) containing pericytes and EC plexus association is required to stabilize the vascular network [57]. Pericytes may arise from differentiation of mesenchymal precursors or the de-differentiation of SMC (see [57] for references). A mesenchymal stem cell (MSC) origin of pericytes is supported by the observation that MSC functionally interact with EC and have pericyte properties [58,59]. In addition MSC are also found in perivascular regions in several adult tissues [60], and form stable, functional vessels when co-cultured in vivo with endothelial precursor cells (ECFC) [61] or terminally differentiated HUVEC which express the pericyte marker, alpha smooth muscle actin, SMA [62,63]. In vitro co-culture of MSC and ECFCs resulted in the rapid assembly of an endothelial network, an increase of CD31, an endothelial marker, and the de novo expression of SMA, in ECFC and MSC respectively. SMA protein expression in MSC requires direct contact between ECFC and MSC. EC/MSC network formation has been shown to be dependent on matrix metalloprotease activity, signaling pathways for VEGF, HGF, and PDGF, and shows increased expression of the ECM protein, fibronectin [64]. Falcon et al. [65] developed a defined media for ECFC/MSC co-culture and demonstrated that endothelial networks formed in basal media were not as extensive or stable as the network driven by VEGF. The VEGF driven network was inhibited by small molecule and antibody inhibitors of VEGF signaling when added during network formation. In contrast, anti-VEGF antibodies did not reduce the size of pre-formed endothelial networks suggesting that the endothelial network transitions into a VEGF-independent state which may be relevant to resistance associated anti-VEGF therapies (see [65] for review). Development of a defined media has enabled the study of alternative pro-angiogenic factors such as EGF, HGF, and FGF in endothelial network, a scenario potentially relevant to the VEGF resistance observed clinically. Interestingly these pro-angiogenic factors in combination or in the presence of VEGF resulted in endothelial networks that could be differentiated by morphology and SMA expression [66].

3.3.2. Neo-vascularization assays for drug screening

Unlike basic research, drug discovery requires that assays provide consistent and quantitative data for efficient compound screening, medicinal chemistry structure activity support, and lead optimization. Drug

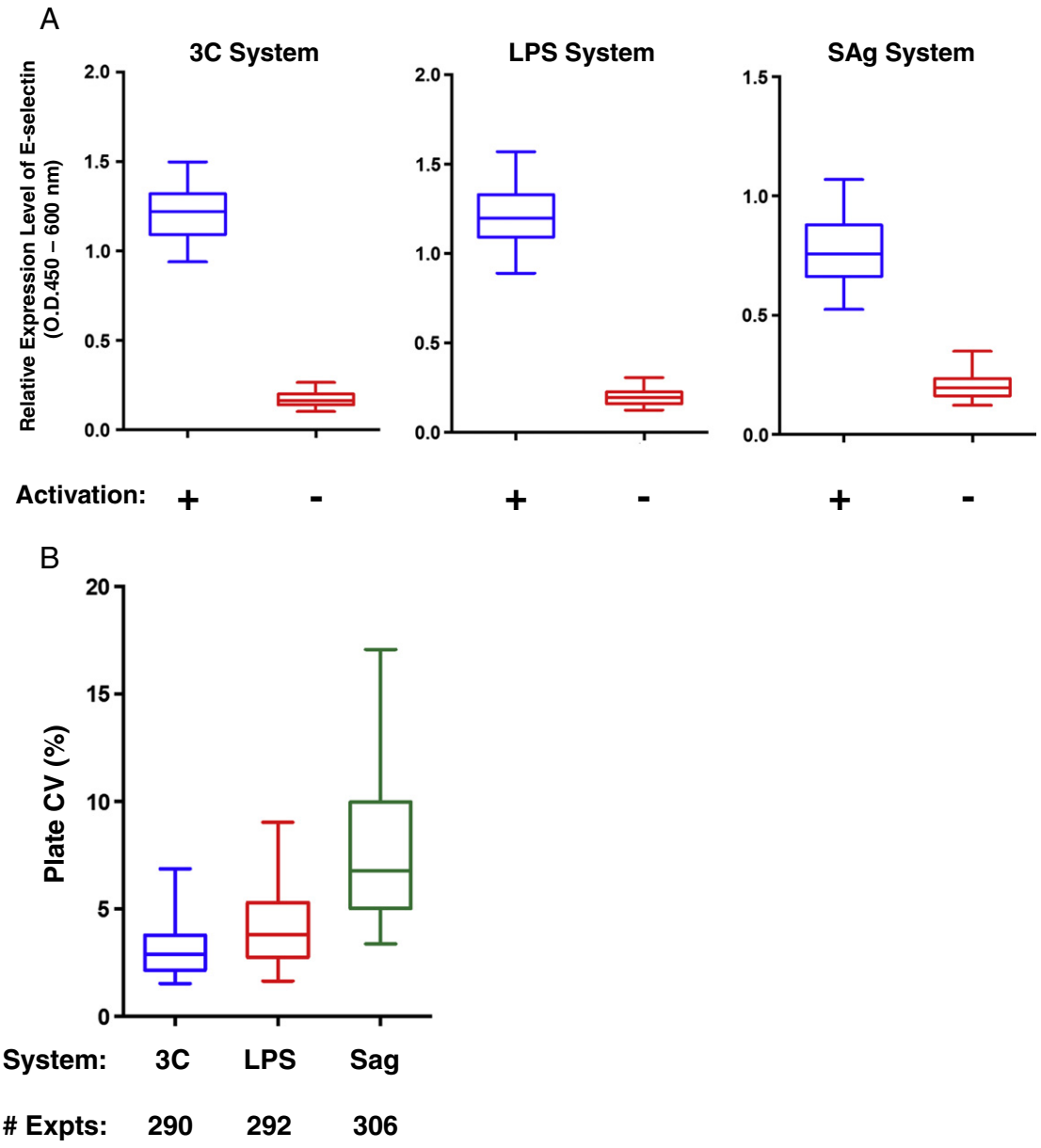

Fig. 3. Historical performance of the BioMAP E-selectin endpoint measurement. A: E-selectin was measured in the BioMAP 3C, LPS and SAg systems under agonist stimulated or activated (+) versus basal unactivated conditions (−) over >5 years and >290 experiments by ELISA. The distribution of measured raw values (O.D. 450–600 nm) is shown as a box plot. B: Distribution of all coefficients of variation (CV, ratio of standard deviation to the mean of 6–8 samples per experiment) of replicate E-selectin measurements of solvent controls in the stimulated condition, for all of the experiments as in A. Experiments were performed as described [10].

discovery assays therefore need to be quantitative and highly reproducible. This requires the enablement robust, statistically validated assays using components that are readily available and of consistent quality, and are compatible with the use of laboratory automation and high-throughput readers. While the EC-matrigel format has been utilized for years to dissect molecular details of endothelial mediated angiogenesis (see above and [49] for review), it is limited for industrial drug discovery by the physical properties of the extract, uncertainty and variability of matrix associated growth factors, and the variable thickness of the resulting 3D matrix which limits the use of automated imaging instruments. Co-culture systems with endothelial cells and either fibroblasts or smooth muscle cells adds the biological richness of a stromal component, as described above and provides an experimental system that can be measured by high-throughput automated imaging [52,53,67]. However, the limited passage potential of primary cells imposes practical restrictions on the number of cells available for testing and potentially adds assay variability, factors which limit assay capacity and statistical robustness.

In contrast, endothelial colony-forming cells (ECFC) and mesenchymal stem cells (MSC) are readily propagated [58,61] so that multiple donors provide sufficient material to build large stocks of frozen cells after limited passage. ECFC and MSC also have progenitor cell characteristics, peri-endothelial MSC take on pericyte characteristics, and in vivo implants containing a mixture of both cells types leads to the formation of functional vessels [58,61,62,64–66]. These observations suggest that the ECFC–MSC co-culture system captures attributes of physiologically relevant processes related to neo-vascularization, a very important feature when considering an experimental system for use in phenotypic drug discovery where the investigator seeks to mimic the therapeutically relevant in vivo environment as close as practical [4,8,68].

These attributes of the ECFC–MSC co-culture facilitated the enablement and statistical validation of a robust 384 well angiogenesis assay [8] which was utilized to screen 32,000 initial compounds

and ~10,000 additional compounds identified by structure based hit expansions of active compounds [68]. Such empirical, functional lead generation strategies interrogate the physiologically relevant biology directly in a molecular target agnostic manner and without prior hypotheses on biological mechanisms [4,8]. This phenotypic screen led to the identification of compounds that are structurally and mechanistically different from marketed standard of care (SOC) drugs, inhibit in vitro neo-vascularization with potencies comparable to SOC drugs, and show activity in vivo [68]. Although at an early stage, these results validate the usefulness of this approach.

4. The tumor microenvironment and primary human cell based co-culture models

The tumor microenvironment (TME) is a complex, heterogeneous tissue where various cell types reside within or in the vicinity of the tumor. Broadly speaking this includes genetically transformed tumor cells, often exhibiting mutational heterogeneity, and the surrounding non-transformed host stroma composed of immune cells, mesenchymal stem cells, endothelial cells, pericytes, fibroblasts, adipocytes, extracellular matrix, and basement membrane [69–72]. In addition to this compositional complexity the TME can change and evolve: immune and mesenchymal stem cells can migrate in to the TME, primary cellular components (tumor and immediate stroma) of the TME can undergo activation and/or differentiation (e.g. to myoepithelial cells), and secondary TME components such as endothelial cells and adipocytes can take on roles to support vascularization and tumor metabolism, respectively. Cellular communication within the TME is multi-factorial and poorly understood involving complex networks of secreted factors in addition to cell–cell contact. Therapeutically the TME is of interest because of its role in promoting tumor cell growth, epithelial–mesenchymal transition (EMT), metastasis, migration, extravasation, and survival of tumor cells in the circulation. In contrast the tissue selectivity of metastasis and observed micro-metastases observed with some cancer types indicates that the tissue/cellular microenvironmental "soil" is not always receptive for growth and support of the transformed tumor cell "seed" suggesting that profound changes in the basic biology of the TME occurs in order to switch between the support or suppression of tumor growth. Clearly there is a rich diversity of biological processes that undoubtedly involves the 3D architecture of the TME. However, 2D co-culture formats can also greatly contribute towards elucidating the complex paracrine and cell–cell interactions contributing to TME differentiation and function. In this section we provide examples of 2D and 3D co-culture studies investigating the TME.

4.1. TME crosstalk: General phenotypic changes

There are a number of studies now describing novel cancer co-culture assays, showing influence of tumor cells on host cells and vice versa [73–82]. Romer et al. (2013) have shown how primary breast cancer cells, when cultured with normal primary human mammary fibroblasts, in a 3D collagen matrix, revert from a disorganized phenotype back to a baso-apical polarized phenotype with more normal differentiation markers [75]. Interestingly another group showed that primary human adult lung cancer associated fibroblasts, when co-cultured with bronchial epithelial cells in a 3D air–liquid interface model, unlike normal fibroblasts, promoted invasion of the collagen gel by the epithelial cells and did not facilitate the formation of a respiratory surface epithelium [76].

Rajski and co-workers (2012) characterized the gene expression profile of breast cancer cell lines when co-cultured with primary human osteoblasts and identified an interferon response gene signature that when tested on patient samples, was associated with lower survival rates. Another gene signature involving IL-6 pathway was associated with time to bone metastasis [77]. In an adipocyte-ovarian tumor cell model, another group also found bidirectional effects of each cell type with increased lipolysis in adipocytes and beta oxidation in the cancer cells suggesting that adipocytes may act as an energy source for the cancer cells [78]. This work also led to the identification of a role for FABP4, adipocyte fatty acid-binding protein, in ovarian cancer metastasis. In another co-culture model of primary mesenchymal stem cells with breast cancer cell lines, a positive feedback loop between the two cell types involving hypoxia-inducible factors (HIFs) and placental growth factor (PGF) and its receptor vascular endothelial cell growth factor receptor I (VEGFR1), that promotes cancer metastasis [79].

4.2. TME crosstalk: Drug resistance

Receptor tyrosine kinase (RTK) inhibitors are a validated class of oncology agents but are frequently limited in clinical utility by acquired or innate drug resistance; Wilson et al. demonstrated that the sensitivity of cancer cell lines to RTK inhibitors can be diminished by simple exposure to one or more RTK ligands [83]. Activation of alternative signaling pathways to bypass/mitigate pathways modulated by a given RTK inhibitor may occur by tumor autocrine effects, stromal paracrine effects, cell–matrix interactions, or tumor stromal interactions (see [84] for review). Strausseman et al. developed a co-culture system to systematically evaluate the ability of 23 stromal cell types to influence the innate resistance of 45 cancer cell lines to 35 anticancer agents and found that stroma mediated resistance is common particularly to targeted agents (65% of tested agents) [85]. Further studies with RAF inhibitor PLX4720 on BRAF(V600E) melanoma cell lines identified stroma derived HGF as a mediator of RAF inhibitor resistance in vitro, an observation which correlated to poor clinical response of patients with BRAF(V600E) melanoma [85]. Significantly, treatment of normal primary fibroblasts by genotoxic chemotherapeutics leads to widespread increased gene expression of secreted proteins, one of which, WNT16B, was further studied and demonstrated to impart resistance to cytotoxic chemotherapy in vivo [86]. Taken together these results emphasize the importance of the stromal environment to chemotherapy resistance of tumors and as suggested by McMillin et al., [84,87] underlines the importance of incorporating appropriate in vitro and in vivo testing paradigms into preclinical research.

4.3. Inflammatory cells of the TME

Tumor associated macrophages (TAMS) interact and sculpt the function of the TME through multiple complex, molecular mechanisms; it is not our intent to review this vast literature but simply to provide a sampling of the complexity of the interaction. TAMs contribute to the complex TME milieu by secreting a number of tumor promoting factors including growth factors, chemokines, pro-inflammatory cytokines, immune modulators, and proteases (see [88–90] for reviews). Tumors secrete chemotactic factors to attract various inflammatory and immune cells into the TME and macrophages can represent a significant portion of the tumor mass [89]. Within the TME, TAMS are associated with tumor cell processes such as enhanced proliferation, migration, invasiveness, and EMT status [91–94] and tumor promoting processes such as enhanced angiogenesis and down regulation of adaptive immunity [89]. The role of TAMs in these various functions suggests the existence of at least four TME sub-categories, related to tumor cell invasion, perivascular sites, stromal regions, and hypoxic/necrotic areas where specific interactions and programming may differ between tumor functional/structural niches [90].

Progress in mimicking aspects of the immune and stromal components of the tumor microenvironment has been made with tumor cell-line host primary human cell co-cultures (Alison O'Mahony, BioSeek, a division of DiscoveRx, manuscript in preparation, personal communication, Figs. 4 and 5). Mixed co-cultures containing an adenocarcinoma colon cancer cell line with primary human fibroblasts and peripheral blood mononuclear cells (PBMC), with or without activation by T cell receptor (TCR) stimulation, were established to evaluate potential tumor-host and/or host-tumor effects (Fig. 4). As shown in Fig. 5,

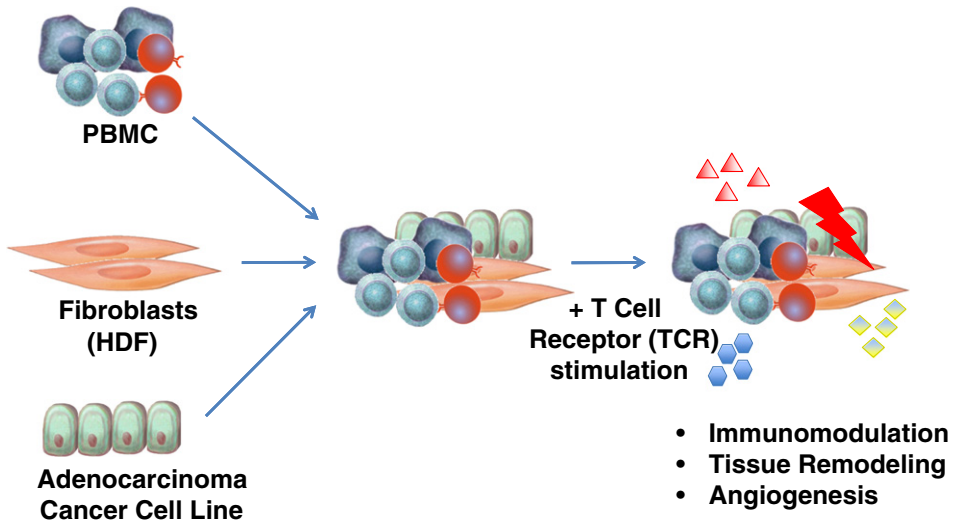

Fig. 4. Construction of BioMAP oncology systems containing tumor host mixed co-cultures. Mixed co-cultures are constructed by combining human peripheral blood mononuclear cells (PBMC) with primary human fibroblasts (HDF) and cancer cell lines. T cells in these mixed cultures are stimulated with the addition of superantigens (SAg) that crosslink the T cell receptor (TCR) and activate the system.

addition of the tumor cell line to the cultures results in reduced production of IFN-gamma and granzyme B, consistent with known immunosuppressive effects of certain tumor cell types (Fig. 5, top two panels). Granzyme B is a serine protease produced by activated cytolytic T cells (CTL), which comprise one of the central effector mechanisms of anti-tumor immunity. As shown in the bottom left panel of Fig. 5, tumor cell-derived production of VEGF, the angiogenic growth factor, is reduced when T cells in the mixed cultures are stimulated, illustrating a protective mechanism of normal host cells on tumor cells. These mixed cultures also show increased IL-17A in the activated co-cultures that contain tumor cells (Fig. 5, bottom right panel). Interestingly an IL-17 paracrine network has been shown to promote tumor resistance to anti-angiogenic therapy [95]. Such mixed co-cultures therefore enable simultaneous assessment of both tumor-host and host-tumor effects, and will be useful for characterization of cancer drugs and drug combinations.

4.4. Fibroblasts in the TME

Fibroblasts contributing to the tumor stoma have been termed peritumoral fibroblasts, reactive stroma, myofibroblasts, carcinoma-associated fibroblasts (CAFs), or tumor-associated fibroblasts. Like the inflammatory and immune cells recruited to the TME, CAFS contribute to the growth factor-cytokine milieu but in addition, greatly influence the cellular and physical properties of the TME and ECM (see [96–101] for reviews). Due to their potential role in regulating the tumor

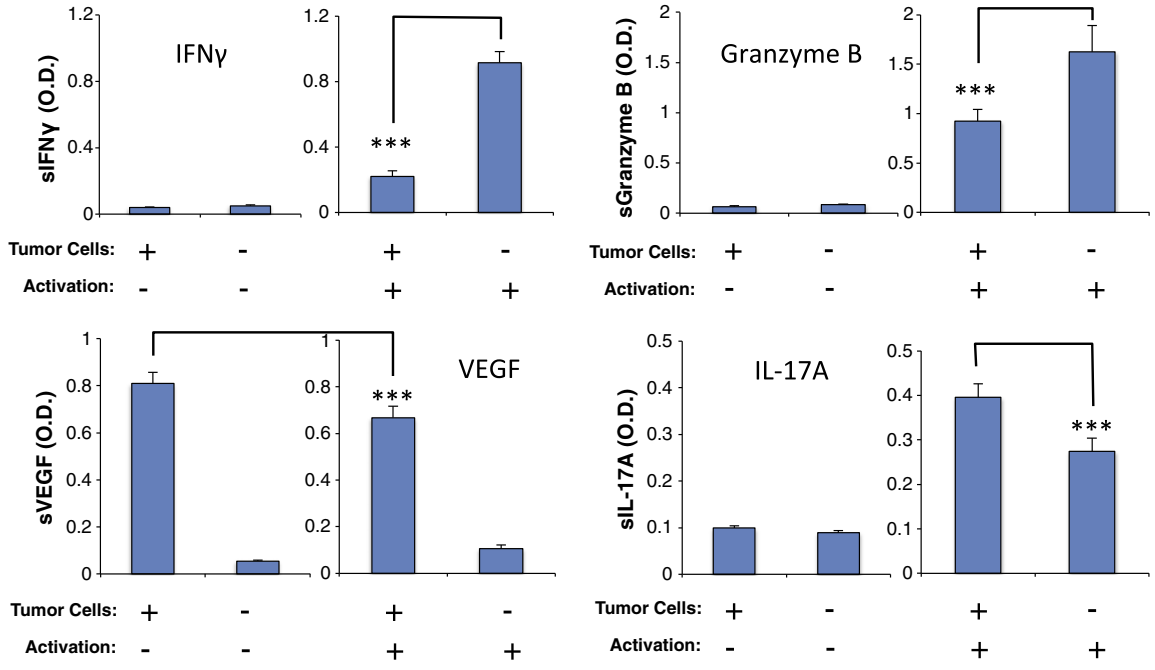

Fig. 5. Mixed co-cultures of primary human fibroblasts and peripheral blood mononuclear cells (PBMC) with or without the addition of adenocarcinoma colon cancer cells (Tumor Cells) or TCR stimulation of T cells (Activation) influences the levels of IFN-gamma (top left), granzyme B (top right), VEGF (bottom left) and IL-17A (bottom right) protein levels. Differences with $p < 0.001$ are indicated (***). Measurements were performed as described [126].

microenvironment, and possibly tumor progression, CAF are an attractive target cell type for the development of phenotypic drug discovery assays, and co-culture assays that may capture CAF regulatory functions would be particularly attractive to pursue. Normal fibroblasts synthesize structural components and proteases in the ECM and are involved in development, stroma homeostasis, and wound healing. CAFs are proximal to tumor cells appearing as large spindle shaped cells with stress fibers with increased expression of cellular proteins, alpha smooth muscle actin (SMA) and fibroblast activation protein (FAP) and various ECM components, including tenascin-C and fetal fibronectin splice variants (see for [102] review). In addition, myofibroblasts from various sources are a relevant source of various growth factors, cytokines and chemokines (see [99] for a review) with certain CAF types expressing a pro-inflammatory mRNA profile [103]. Interestingly, the origin of CAF cells is not entirely clear, various studies have suggested that CAFs may originate from fibroblasts, smooth muscle cells, mesenchymal stem cells, endothelial cells, epithelial cells, pericytes, and adipose (see reference [97] for a review). Regardless, the overall myofibroblast and CAF literature underlines the potential roles of these cell types and gene products to modulate tumor cells, infiltrating immune/inflammatory cells, and other resident TME cells using a multitude of complex mechanisms related to cell–cell, paracrine and autocrine interactions, and stroma remodeling [96–102].

Primary CAFs can be extracted from various tumor types, isolated, and grown in limited passage [104,105]; CAFs isolated from breast cancer patients demonstrate higher SMA staining and contractility than matched fibroblasts isolated from normal patient tissue [105]. Isolated CAFs enhance tumor progression in vivo when co-transplanted with transformed epithelial cells relative to a co-transplant with normal fibroblasts or CAF and tumor cells alone [103–105]. Importantly outgrowths resulting in CAF/transformed epithelial co-transplants, but not the corresponding constructs with normal fibroblasts, resemble neoplastic tissue morphology with a cytological appearance consistent with malignant tumor tissue [104], promote tumor angiogenesis, and enhance mobilization and recruitment of $Sca1^+$ $CD31^+$ endothelial progenitor cells [105] and macrophages [103] to the tumor. Interestingly normal fibroblasts and MSCs take on a CAF phenotype when co-transplanted with tumor cells and enhance tumor size/volume/weights over tumor cells alone [103,106,107]; bone marrow MSCs have also been reported to enhance the metastatic potential of weakly metastatic breast cancer [108]. In vivo lineage tracing studies indicate that >20% of CAFS originate from bone marrow and are derived from MSC in a murine model of inflammation induced gastric cancer [109]. In murine models of ovarian and breast cancer CAFs appear to be heterogeneous and recruited from two distinct sources, bone marrow and adipose derived cells [110]. Taken together these results indicate that factors in the TME lead to the recruitment of precursor cells from distinct tissue sources and facilitate their conversion into stromal CAF which enhances tumor growth and metastasis in vivo.

Increased levels of myofibroblasts or CAFs in tumor stroma results in the over production of collagens, fibronectins, proteoglycans and tenascin C and the formation of fibrotic regions termed desmoplastic stroma. Significantly, increased expression of tenascin C in breast and bladder cancer stroma correlates with poor prognosis [111,112]; increased numbers of stromal myofibrobasts correlate with poor prognosis of breast carcinoma [113] and disease progression of oral cancer [114]; patients exhibiting increased desmoplastic stroma shows an overall lower survival rate for breast cancer [115] and invasiveness of small lung adenocarcinoma [116]. These observations suggest that an agent that inhibits or reverses CAF and/or desmoplastic stroma formation may be a potential therapeutic to decrease tumor growth and metastasis.

Towards this end CAF-like cells have been formed following incubation of MSCs with tumor cell conditioned medium or by MSC-tumor cell direct co-culture [117,118]. In addition, co-cultures of MCF-7 breast tumor cells and immortalized fibroblasts increase the levels CAF protein markers SMA, calponin, and vimentin and ECM components, tenascin C and collagen 1 [119] in the fibroblast population [119]. These studies provide a proof of concept for a CAF differentiation assay which may be amenable to drug discovery in an industrial environment. Fig. 6 illustrates the direct co-culture of human adipose derived MSCs with MCF-7 breast cancer cells in a 96 well format. Expression of SMA is observed in the stromal cells proximal to a MCF-7 cell cluster with low SMA expression in MSC cells distal to the tumor cells (Fig. 6). Additional CAF markers, PDGFRb and tenascin C, and activated fibroblast markers, extra domain A-containing fibronectin (EDA-FN) and SPARC, are similarly increased in MSC-MCF-7 co-culture (Hsu, manuscript in preparation). Statistical validation of a 384 well version of this assay is ongoing and will potentially form the basis of a robust assay platform to investigate CAF biology using small and macromolecule probes.

5. Conclusions/future efforts

An analysis of clinical drug trial outcomes indicates that the majority of failures are due to lack of human efficacy; these observations highlight the importance of developing early drug discovery models with enhanced translation to clinical studies. Towards this end, this review summarizes efforts and provides examples of primary human cell co-cultures in industrial drug discovery and screening. Although essential to drug discovery, development of physiologically relevant assay

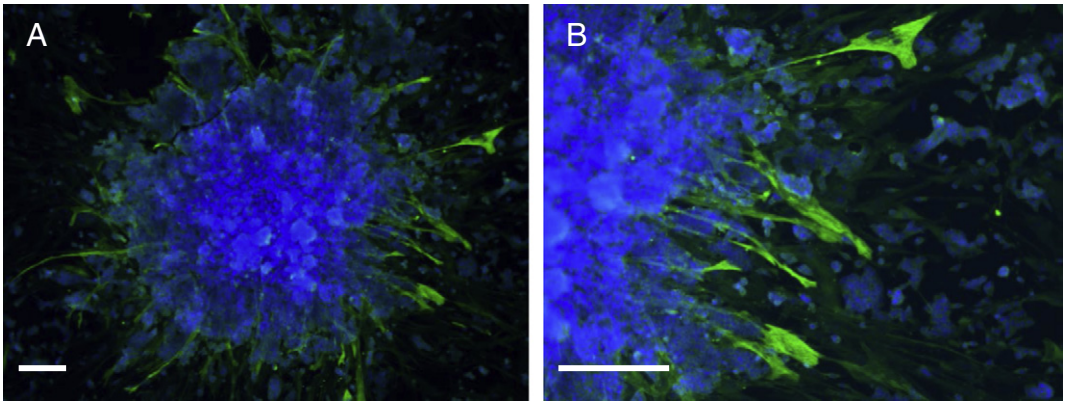

Fig. 6. Induction of a cancer activated fibroblast phenotype in primary cell co-culture. MCF-7 breast cancer cells and human adipose mesenchymal stem cells (MSC) were co-cultured for 6 days, fixed and incubated with antibodies to smooth muscle actin (SMA), a CAF marker (green) and Hoechst, a DNA stain (blue). Fluorescence microscopy of co-culture: A at 10×; B at 20×; white bar corresponds to 50 μM. Expression of SMA is observed in the stromal cells proximal to a MCF-7 cell cluster with low SMA expression in MSC cells distal to the tumor cells. Additional CAF markers, PDGFRb and tenascin C, and activated fibroblast markers, EDA-FN and SPARC, are similarly increased in stromal region of MSC-MCF-7 co-cultures (not shown).

systems and the use of primary human cells and co-cultures in industrial processes is challenging. Primary cells can be expensive and require significant infrastructure for managing cell banks and maintaining quality control. The need to pool cells from multiple donors for some assays may preclude personalized medicine applications, as genetic differences would be masked. Development of robust assay endpoints and rigorous statistical assay validation often are difficult and require the use of data normalization methods.

The development of physiologically relevant in vitro assay systems for industrial scale high throughput screening is challenging with many potential scientific avenues to explore and only a few success stories. In this review we have focused on primary human cell 2D co-culture models since they are relatively amenable to HTS, have proven utility, and can capture aspects of the functional and compositional heterogeneity–complexity found in the cellular-tissue microenvironment in vivo. In addition to the inflammation, angiogenesis, and the tumor microenvironment related systems discussed here, increased access to primary cell and/or stem cell derived cells is expected to enable development of co-culture systems relevant to additional disease biology areas such as central nervous system, endocrine disorders, and others.

Significantly, 2D co-culture systems are just a beginning. Technology to provide differentiated cell types from patient derived iPS cells [23,25], advances in highly multiplexed assay, imaging, and cytometry technologies [120], the availability of tissue culture in microfluidic and hemodynamic flow environments [121,122], and technologies for 3D cellular printing and cell culture [123,124], such as those described elsewhere in this issue, provide a scientific/technical tool box for future development of physiologically relevant in vitro assays. These advances in cell biology, bioengineering, and assay technologies coupled with efforts to miniaturize and mass produce 3D cultures [125], will increase the physiological relevance of industrial drug discovery screening assays.

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