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Integration of systems biology with organs-on-chips to humanize therapeutic development

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ABSTRACT

"Mice are not little people" – a refrain becoming louder as the gaps between animal models and human disease become more apparent. At the same time, three emerging approaches are headed toward integration: powerful systems biology analysis of cell-cell and intracellular signaling networks in patient-derived samples; 3D tissue engineered models of human organ systems, often made from stem cells; and micro-fluidic and meso-fluidic devices that enable living systems to be sustained, perturbed and analyzed for weeks in culture. Integration of these rapidly moving fields has the potential to revolutionize development of therapeutics for complex, chronic diseases, including those that have weak genetic bases and substantial contributions from gene-environment interactions. Technical challenges in modeling complex diseases with "organs on chips" approaches include the need for relatively large tissue masses and organ-organ cross talk to capture systemic effects, such that current microfluidic formats often fail to capture the required scale and complexity for interconnected systems. These constraints drive development of new strategies for designing in vitro models, including perfusing organ models, as well as "mesofluidic" pumping and circulation in platforms connecting several organ systems, to achieve the appropriate physiological relevance.

Keywords: organs-on-chips, 3D liver culture, perfusion, drug development, inflammation, organ crosstalk, tissue chip, intestine

1. INTRODUCTION

1.1 Complex diseases require new approaches

Drug development for cancer, and particularly for chronic inflammatory diseases (e.g. diabetes, arthritis, endometriosis, and Alzheimer's), which typically have weak genetic linkages and poorly-understood, systemic mechanisms where damage accrues over many years, is notoriously difficult. While drug safety failures are typically highlighted in the organs-on-chips literature [1, 2], lack of efficacy is currently the major cause of drug failure overall, and failure rates are higher for complex diseases that have no clear single genetic basis [3]. Such diseases are highly prevalent. For example, the most common cause of abnormal liver function tests in western countries is non-alcoholic fatty liver disease (NAFLD), which has a prevalence of 20-50% [4, 5]; is often co-morbid with Type 2 diabetes; affects tens of millions of individuals worldwide; and is now the second leading cause for liver transplants in the US [4, 5]. With many different possible targets in the disease mechanism, and clinical heterogeneity in patient disease characteristics, over a

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dozen new drugs are in the pipeline or clinical trials, joining several established accepted and off-label treatments that have yet to "cure" the symptoms most patients experience [6-8]. Further, at least 10% of reproductive-age women worldwide experience debilitating pain and infertility due to non-malignant growth of endometrium outside the uterus (endometriosis) or displaced into the uterine muscle (adenomyosis) [9, 10]. Current FDA-approved medical therapies attempt to put brakes on estrogen, but a substantial fraction of patients either fail to respond to drugs or suffer severe side effects; surgical removal of lesions helps only a fraction of patients [9-11]. Optimism that new classes of anti-inflammatory drugs developed for other chronic inflammatory diseases such as arthritis has been tempered by the unsuccessful outcomes of recent clinical trials [12], despite the positive outcomes in pre-clinical animal models of disease [13-15].

These highly prevalent diseases are examples of how highly heterogeneous patient population and poorly-representative animal models work together to stymie both development and clinical translation of effective new therapies [4, 5, 16]. One part of the problem in developing effective drugs for such diseases – defining common mechanistic themes among subgroups of patients - is being tackled by innovative integration of multiple –omics measurements across the scales of information flow in cells, from DNA to RNA to protein, protein activity states, and metabolites [17], as well as similar types of analysis of patient-derived immune cell function [18]. Such approaches led to the first molecular classification of endometriosis patients and insight into the lack of efficacy in JNK inhibitor trials [11, 19] and are yielding insights into type 2 diabetes [17, 18].

Another part of the problem – relative inability to carry out mechanistic studies of new therapies in patients, and lack of animal or in *vitro models* – is still a major bottleneck. This gap underscores the need for bold new approaches to model these diseases *in vitro*. Unfortunately, cell culture models, including those derived from human pluripotent stem cells (PSCs), are inadequate: not only do they fail to capture crucial heterogeneous cell interactions within a single organ, development of chronic inflammatory diseases is often the convolution of genetics with lifestyle and environmental exposures, including infection, that are integrated into epigenetic modification of cells throughout the body [17, 20]. Clearly, one step is to develop more complex individual organs-on-chips that capture the local features of disease, especially inflammation. But for systemic diseases affecting multiple organs, organ-on-chips platforms designed to capture physiological interaction phenomena between critical organs systems may yield improvements in understanding both efficacy and off-target effects. Here, we describe examples of how organs-on-chips are being deployed to model complex disease states, and highlight technical challenges in merging disparate fields to design, implement and interpret experiments.

1.2 Dormancy and growth of metastatic cancer: a challenge beyond the scope of traditional microfluidics?

The overwhelming majority of breast cancer patients present with no evidence of distant metastatic disease; yet, following removal of the primary tumor and prophylactic chemotherapy to kill disseminated tumor cells throughout the body, a significant fraction will develop tumors years later in the bones, liver, lungs, and brain and succumb to metastatic disease [21]. Triple negative breast cancer (i.e., lacking expression of estrogen, progesterone, and Her2 receptors) is

particularly deadly: about 25% of patients die from metastatic disease within 5 years of diagnosis despite aggressive prophylactic chemotherapy [22]. What makes disseminated tumor cells resistant to chemotherapy? What makes these dormant tiny metastatic clusters wake up and grow? These questions cannot be studied in patients – the metastases are not visible -- and animal models fail to capture the key features of human responses, including the increasingly-appreciated role of the human immune system in progression [22, 23].

Numerous 3D models of tumor cells in isolation have been developed in the past few decades [24], with a notable recent emphasis on how the mechanical microenvironment and geometry of multicellular tumor aggregates influence malignant properties [25]. Similarly, models have emerged to capture tumor-stroma interactions [26]. Microfluidic systems have been deployed to control oxygen and growth factor microenvironments [27, 28], tumor-endothelial interactions [29-31] and events in passage of tumor cells from micro-vessels into surrounding tissue[32, 33]. While each of these systems captures subsets of tumor behaviors, a significant gap exists in addressing how metastatic nodules of up to several hundred microns interact with human host tissue.

Can microfluidic devices, which typically support culture of tens of thousands of cells, capture the complexity of metastatic resistance to chemotherapy drugs, and the signals that wake up dormant tumors? A single adult human liver lobule comprises about 10^7 cells [34] and initial metastases are estimated to range from single cells up to a few tens of cells [35]. Thus the ratio of host to metastatic cells in a traditional microfluidic device is substantially skewed, and are more representative of primary tumors or end stage metastases [29, 36]. This scaling mismatch may skew tumor cell behaviors, which *in vivo* are regulated by the overwhelming host signaling milieu and innate inflammatory response [37-39].

Disease modeling problems such as micrometases that require "meso-scale" tissues, comprising hundreds of thousands to many millions of cells, present interesting challenges in terms of integrating tissue engineering with appropriate pumping and fluidics to provide adequate perfusion rates to such structures, particularly in a user-friendly platform format. Based on the oxygen consumption rate of liver, for example, flow rates of 6-10 μL per million cells per second are needed just to deliver oxygen if cell culture medium is the circulating fluid [40-42]. Another often under-appreciated facet of disease modeling with implications for device design is that inflammation – which is a crucial part of a vast number of diseases lacking adequate therapies – is strongly regulated by steroid hormones [43, 44], which partition strongly into elastomers such as polydimethylsiloxane (PDMS), making their concentrations difficult to control [45].

In order to address these constraints, we developed a 3D micro-perfused liver model aimed at supporting long-term culture of 3D liver-like tissue at scales over a million cells, in a user-friendly format. The heart of the system is a thin (~0.25 mm) scaffold perforated with an array of ~0.3mm diameter channels situated on a membrane support and maintained in a re-circulating flow multi-well plate bioreactor[40-42, 46]. Liver cells seeded into the scaffold form 3D tissue-like structures, which are perfused at flow rates sufficient to create a physiological oxygen tension drop across the scaffold without excessive shear [40, 47] and which can be maintained in a functional state for weeks in serum-free culture medium. The reactor system is micro-machined from polysulfone and recirculation is driven by on-board microfluidic pneumatic pumps that are programmed with a user-friendly interface, innovations that are crucial for carrying out

quantitative pharmaco-kinetic (PK) studies, ease of use in diverse laboratory settings, and adaptation to multi-organ formats. The performance of this system in the context of other liver reactors has been recently reviewed [42, 48, 49].

Although we and others have extensively characterized the PK of common small molecule drugs by primary human liver cells in this reactor system [50-52], standard culture systems are reasonably effective for most small molecule drug PK assays [42], thus, the kinds of pre-clinical assays driving potential use of this system in the later stages of drug development generally involve the immune system. For example, we recapitulated a complex immunologically-based drug-drug interaction between the anti-IL6 receptor antibody tocilizumab and the metabolism of simvastatin - a phenomenon that could not be reproduced in standard cultures [46]. Inflammation drove dose-dependent suppression of CYP450 activity and metabolism of simvastatin, along with increased production of C-reactive protein (CRP) and cell-produced cytokines and cytokine receptors [46]. Importantly, these responses depend on using physiological levels of cortisol, a steroid hormone, rather than the excessively high concentrations used to boost CYP450 activity in typical hepatocyte cultures [43, 44]. This multi-well plate reactor system has also been applied to model NAFLD, including fat accumulation, increased production of adipokines, and suppression of CYP450 activity – which were all modulated by treatment with known drugs [53].

A potential advantage of the scale of this 3D perfused liver microreactor system is to first establish micro-metastases in the context of a relatively large ($\geq$ 1 million cells) mass of liver cells, and then to analyze complex cell-cell communication network signatures using both measurements that can be routinely made in patients (on the circulating medium) as well as measurements that cannot also be made on patients – the kinetics of tumor cell growth and death. An outstanding challenge in targeted chemotherapeutics is the emergence of resistance to chemotherapy, particularly to inhibitors of specific kinase or growth factor pathways – the tumor initially responds, but surges back after time [23]. Mechanistic systems biology analysis of how intracellular and extracellular signaling networks are connected, probed using in vitro models, have recently been linked to patient outcomes via measurements of proteins shed by tumor cells that appear in the plasma of patients [23]. These models allow some prediction of how patients will respond (or not) to therapies based on a compendium of measurements before and soon after treatment with the drug [23].

As a first important step to model triple negative breast cancer (TNBC) micrometastases in liver, we established the 3D liver model using primary human hepatocytes and non-parenchymal cells, and seeded the cultures with 100-500 fluorescently-labelled MDA-MB-231 cells as a model TNBC cell line [36, 54-56]. Micrometases were established even with the lower limit of cell seeding. Although these cells are typically highly proliferative in standard culture, MDA-MB-231 cells were quiescent in the context of the 3D liver tissue in the microreactor format – in other words, they exhibited hallmarks of dormancy [36, 54-56]. A comprehensive analysis of cytokine and growth factor networks using this model showed that a few tumor cells re-wire the entire culture, and that shifting to a hydrogel scaffold for supporting liver cells provides a more quiescent background liver state than the standard scaffold [54-56]. Interestingly, under standard medium conditions, tumor cells in contact with stiff polystyrene scaffolds are proliferative and responsive to the standard chemotherapy drug doxorubicin, while tumor cells in the 3D tissue are quiescent and relatively resistant [36, 54-56].

One potential mechanism for waking dormant tumor cells is inflammation in the liver, which may arise due to increased permeability of the gut from infection, drugs, alcohol, or other causes [44]. As tumor cells and the microenvironment can respond to inflammatory cues [36], leaky gut may lead to stimulation of dormant micrometastases. To model this, we adapted the Liverchip platform to link a transwell gut module with the liver module, using the same on-board pumping technology to drive fluidic communication between the two modules as well as circulation within each module. A gut MPS with features of innate immunity was created by seeding a 9:1 mix of absorptive enterocytes (CC2BB/e1 line) and mucin-secreting goblet cells (HT29-MTX line) (adapted from [57]) on the apical surface and dendritic cells, obtained from *in vitro* differentiation of human PBMCs-derived monocytes, on the lower side of the membrane. After 21 days, the epithelial layer exhibited trans-epithelial electrical resistance (TEER) values > 250 $\Omega \bullet cm^2$, produced mucus (as assessed by Alcian blue [58]), and was penetrated by extensions of dendritic cells. These interconnected organ systems show synergistic responses to inflammation [59] and establish a model for future studies of gut-derived inflammation on cancer micrometases in liver.

1.3 Frontiers in multi-organ systems: integrating minimal physiological models with hardware

Multi-organ platforms are just beginning to emerge as potential tools for disease modeling and drug development, and many challenges remain: first, in defining where the information from such systems justifies the added cost, as each organ system comprises a mix of expensive primary human cells, and second, in defining the appropriate "minimal set" of interacting organ systems to represent a disease state. Existing multi-organ platforms that might be used for disease models often fail to meet requirements for quantitative PK due to use of polydimethylsiloxane (PDMS) and highly constrained pumping or fluid transport schemes [60, 61], large circulation volumes that preclude detection of metabolites and cell-produced proteins [62, 63], or are geared more toward pre-clinical tests of metabolism and toxicity and thus lack organ complexity [1, 2]. Hence, there is tremendous room for innovation in integration of the hardware, biology, and modeling.

A crucial link in this regard is the emerging field of Quantitative Systems Pharmacology (QSP). QSP combines experimental and computational approaches to pharmacological concepts [64, 65] and is being extended to design and interpretation of MPS technologies [50, 66-68]. While a variety of pharmacokinetic and pharmacodynamic (PKPD) models are used to define relationships between drug kinetics and biological effects *in vivo*, PKPD models for MPS analysis must capture the interrelated physical dynamics (e.g., flow rates in the MPS) and biological dynamics (e.g., cytokine/growth factor/hormone production and release) in order to select the appropriate experimental conditions, analyze results, and predict human outcomes. I.e., they must use ***physiologically-based*** PK (PBPK) models. *In vitro* to *in vivo* translation (IVIVT) is an interpretive step that compares and validates MPS results to clinically-relevant outcomes. While *in vivo* to *in vitro* correlation (IVIVC) and *in vivo* to *in vitro* extrapolation (IVIVE) methods have been widely used to predict PK, IVIVT goes a step further to include analysis of endogenous growth factor, inflammatory and hormone signals that affect function. Thus, IVIVT approaches can additionally predict PD, clinical toxicology, biomarkers, and patient stratification using information from MPS technologies. PBPK models for IVIVT can

quantitatively forecast human responses, accounting for missing organs, organ and media size mismatches, and drug exposure.

2. CONCLUSION

2.1 Application-based approaches

There are no 'one size fits all' solutions for modeling complex human disease "on a chip", and each model will need to be tailored to its intended application, capturing the minimum biological complexity in order produce translatable biological outputs. Tackling the challenges of integrating multi-organ systems in a way that yields meaningful advances will therefore require bringing together groups with diverse expertise in biology, engineering, and systems pharmacology.

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