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Review

Harnessing organs-on-a-chip to model tissue regeneration

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SUMMARY

Tissue engineering has markedly matured since its early beginnings in the 1980s. In addition to the original goal to regenerate damaged organs, the field has started to explore modeling of human physiology "in a dish." Induced pluripotent stem cell (iPSC) technologies now enable studies of organ regeneration and disease modeling in a patient-specific context. We discuss the potential of "organ-on-a-chip" systems to study regenerative therapies with focus on three distinct organ systems: cardiac, respiratory, and hematopoietic. We propose that the combinatorial studies of human tissues at these two scales would help realize the translational potential of tissue engineering.

INTRODUCTION

In the early years of the field, tissue engineering was focused on the development and eventual application of lab-grown tissues for regenerative medicine, using a variety of cell sources, materials, and culture systems. More recently, human "organs-on-a-chip" (OOCs), also known as microphysiological systems (MPS), have emerged as an entirely new approach to recapitulate organ physiology during development and disease (Figure 1). These models utilize human iPSC-derived or primary cells and span a range of technologies, from self-assembling organoids to bioengineered tissues and microfluidic organs-on-a-chip (Bhatia and Ingber, 2014; Hofer and Lutolf, 2021; Ronaldson-Bouchard and Vunjak-Novakovic, 2018). In this review, we refer to these technologies as OOCs unless specified differently.

An increasing number of groups are now developing OOC models of the heart, lung, liver, marrow, kidney, gut, and brain, among other organ systems, in the form of minimally functional tissue units capable of modeling *in vitro* some aspects of human physiology in health and disease, as well as the effects and mechanisms of drug actions (Low et al., 2021; Sharma et al., 2020).

From early works that have used decellularized bone grafts to recent stem cell therapies, regenerative engineering has also faced numerous challenges to reach the clinic. Biomaterials, either with or without cellular sources, have been employed to promote regeneration of some but not all organs, with skin, bone, and vessels being largely successful (Amini et al., 2012; Chen et al., 2020; MacNeil, 2007; Pashneh-Tala et al., 2016). There have been major advances in using human cells, primary and iPSC-derived, to help guide regenerative function in various organ systems. Major challenges in this area, including large-scale cell production, effective cell engraftment, and tissue vascularization, are currently being addressed through new bioengineering approaches (Fleischer et al., 2020).

We discuss progress in two complementary areas of tissue engineering, OOC models and regenerative medicine, that show immense potential for convergence and synergy (Figure 2). Micro-scaled OOCs can be valuable for studying the underlying biology of injury and regeneration, with the aid of real-time monitoring using biosensors and imaging techniques that allow for understanding dynamic changes in cell and tissue functions (Figure 1). OOCs enable researchers to de-couple the effects of cell type, cell-cell, cell-microenvironment, and tissue-tissue interactions, as well as to advance our fundamental understanding of native organ function and the effects of treatments. For example, dosing strategies can be easily manipulated *in vitro* to mitigate or enhance inflammation or to optimize cell dosage in cell therapy applications. We focus here on the potential use of bioengineered models *in vitro* for studying new approaches in regenerative medicine in tandem with animal models, using examples from three organ systems: cardiac, respiratory, and hematopoietic (Figure 2).

Bioengineering human physiological functions on the micro and macro scale

Modeling the biological complexity of the body *in vitro* has been widely pursued, but still remains a difficult challenge. Recapitulating cell microenvironment, cell-cell interactions, and spatial organizations seen in native human tissues brings major challenges. While acknowledging that it is simply impossible for every single biological characteristic to be faithfully represented *in vitro*, the field is trying to answer the long-standing question of "how simple is complex enough"? In the bioengineering community, it is often understood that with higher biological fidelity, we often see more realistic functional outputs, but generally at the expense of longer protocols for tissue assembly and maturation. In some applications, however, establishing just the basic functional outputs can be sufficient, as for *in vitro* studies of toxicity and disease modeling. In contrast to tissue implants, which require stable multi-scale vasculature perfused with blood, OOCs are often able to recapitulate functional validity of an organ system with minimal or no vascularization. Using

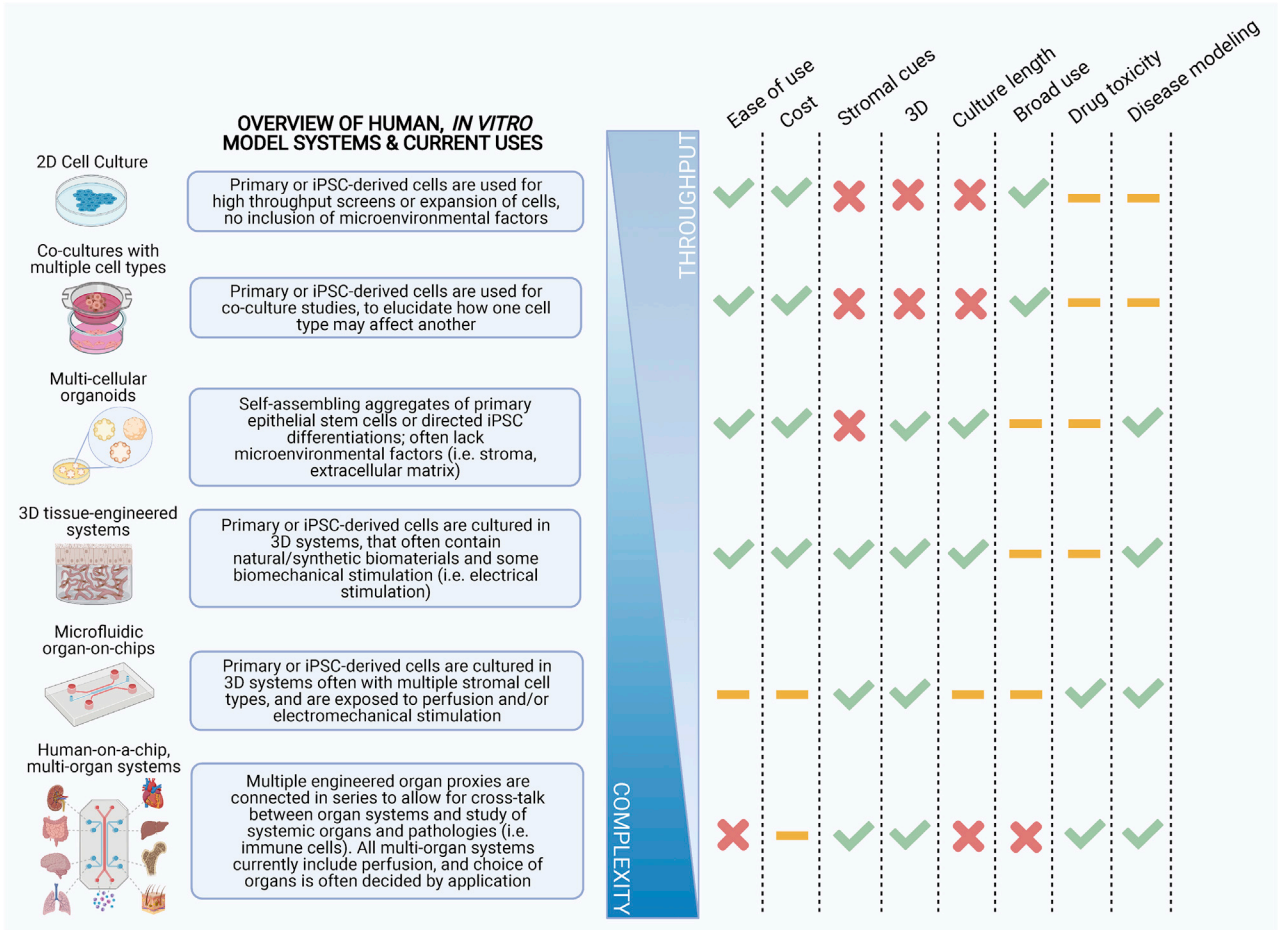

Figure 1. *In vitro* models

A range of models, from 2D cell culture to organoids and human OOC systems, enable a number of applications for modeling drug toxicity, disease phenotypes, and organ function while balancing the inverse relationship between complexity and throughput. Key: highly (green checkmark), somewhat (yellow line), or not (red X) applicable.

patient-derived cells and materials, OOC model systems can help understand the mechanistic differences between the patient groups to study sex differences, identify groups more susceptible to a specific disease, or classify patients into subpopulations to predict prognosis. Other *in vitro* models, like organoids, have emerged over the past decade to study self-organizing tissues from iPSC or primary cell sources, including applications in gut epithelia, the brain, and vasculature (Lancaster et al., 2013; O'Neill et al., 2021; Sato et al., 2009; Wimmer et al., 2019). These models are high throughput and are starting to become commonplace in many research institutions, though they often lack the environmental milieu, controlled spatial organization, and maturity necessary for mimicking adult human physiology (Figure 1). An important goal for the field is to design the bioengineering tools in the context of biological questions of interest.

The field of tissue engineering and regenerative medicine has steadily developed, as shown by the number of papers published in each field each year (Figure 3A). Since the mid-2000s, the advent of iPSCs and encompassing technologies has led to an exponential growth of OOC models (Figure 3B). Going forward, OOCs can provide testing opportunities for understanding the efficacy and safety of regenerative therapeutics in human tissue models and assessing the mechanistic basis of the therapy prior to its testing in human patients (Figures 3B–3H).

Cardiovascular system: Modeling cardiac muscle to advance cardiac regeneration

Cardiovascular diseases are the leading cause of death in the Western world. Over the past few decades, major strides have been made in cardiac tissue engineering to address this clinical need, from early work in understanding the behavior of animal-derived cardiomyocytes to recent developments in vascularizing complex human muscle tissue and deriving ventricular and atrial cardiac cells and tissues (Fleischer et al., 2017; Goldfracht et al., 2020; Ogle et al., 2016). Cardiac muscle is a dense tissue rich in collagen and elastin fibers, containing a variety of cell sub-populations such as cardiomyocytes, cardiac fibroblasts, endothelial cells, smooth muscle cells, and macrophages. While cardiomyocytes are not the largest cell population in the heart, they are the most studied, and they are most critical for cardiac regeneration as the non-proliferative, terminally differentiated

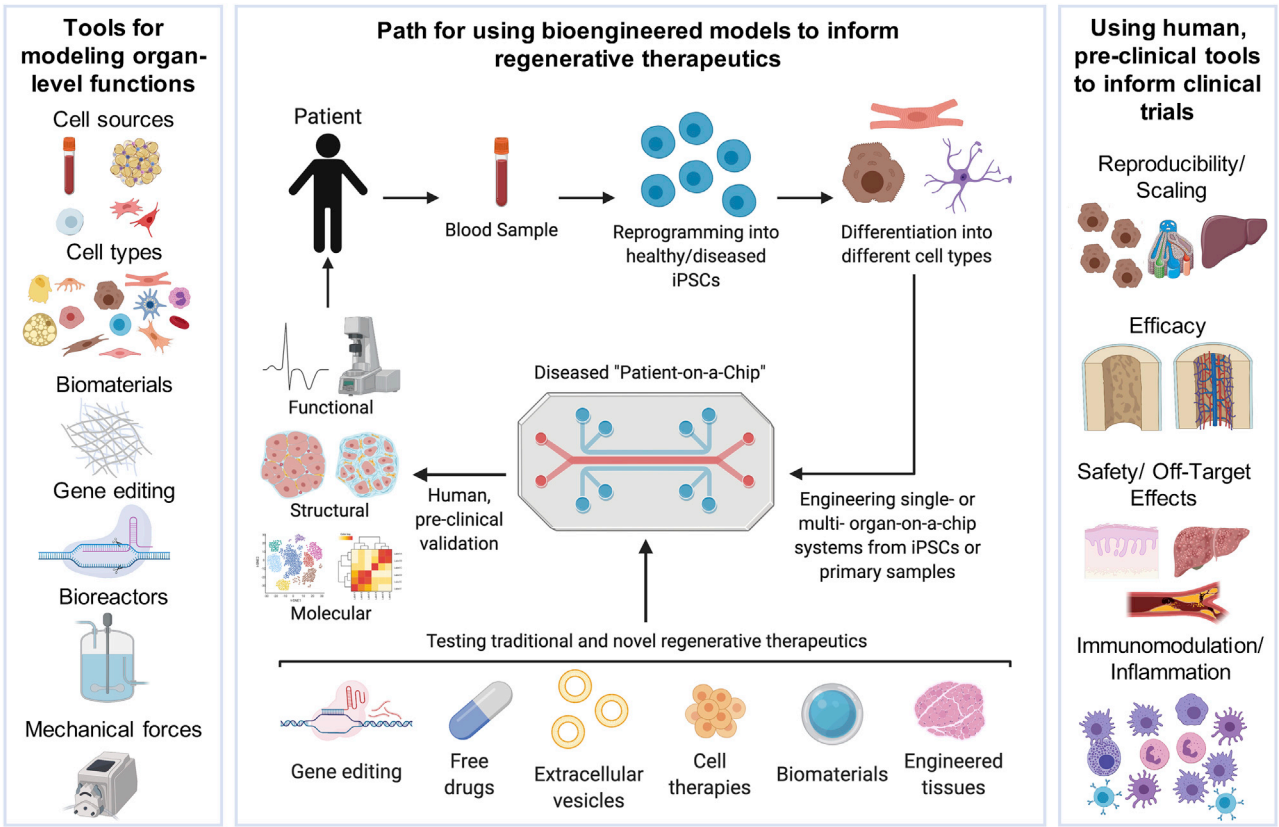

Figure 2. A framework for using engineered human tissues for testing regenerative therapies
Advances in bioengineering tools enable high-fidelity, patient-specific models of different organs and tissues, with potential for studying different therapeutic strategies for translational efficacy and safety testing

cells driving the contractile behavior of the muscle. In the states of injury (e.g., following myocardial infarction) or disease (e.g., cardiomyopathies), these cells are unable to restore the functional capacity of healthy muscle. Following damage, the non-parenchymal cells, mainly fibroblasts, tend to proliferate and activate into myofibroblasts, to further exacerbate fibrotic disease (Kapoun et al., 2004). Despite major advances in the field, there is still a critical need to better understand the underlying molecular mechanisms of disease and develop effective strategies to promote regeneration and restore heart function.

Cardiac tissue engineering

Successful engineering of cardiac muscle began from studying embryonic chick cardiomyocytes seeded within collagen hydrogel and their organization into spontaneously beating tissues in the late 1990s (Akins et al., 1999; Carrier et al., 1999; Eschenhagen et al., 1997). Many groups continued to develop methods for engineering cardiac tissues, using different cell sources (in particular from neonatal rat or chick embryo cardiomyocytes), varying the cell density, and developing advanced biomaterials and bioreactor designs to guide functional tissue assembly. It has been shown that tissues engineered from animal-derived cells can be used both for electrophysiological studies (Bursac et al., 1999) and for transplantation to improve cardiac function in animal models of myocardial infarction (Leor et al., 2000).

External stimuli were recognized as very important for synchronized contractions and maturation of engineered tissues. In some of the first studies using engineered cardiac tissues, Fink et al. demonstrated that mechanical stimulation, including unidirectional cyclic stretch, induced cardiomyocyte alignment and hypertrophy and led to enhanced functional properties of cardiac tissues grown from neonatal rat cardiomyocytes *in vitro* (Fink et al., 2000). Auxotonic mechanical stimulation can be applied by culturing engineered cardiac tissue between flexible pillars or posts, enhancing tissue contractile properties (Boudou et al., 2012; Zimmermann et al., 2006). In separate studies, electrical stimulation designed to induce macroscopic contractions increased cell alignment and coupling, in addition to improving ultrastructure, gene expression, and functional tissue properties (Radisic et al., 2004).

In the mid-2000s, a new age of human stem cell derived cardiomyocytes started with directed ESC differentiation into mesodermal lineages *in vitro* (Laflamme et al., 2007; Yang et al., 2008). Lessons learned from early work using animal-derived cardiomyocytes paved the way toward successful studies in engineering cardiac tissues from ESC-derived sources (Caspi et al., 2007). Recently after, the Kamp and Gepstein groups independently published derivation of functional fetal-like cardiomyocytes from human iPSC sources (Zhang et al., 2009; Zwi et al., 2009). Following differentiation, these cells were seeded into 3D scaffolds, co-cultured with other cell types such as MSCs, ECs, or epicardial cells (Bargehr et al., 2019), and subjected to external stimulation to promote maturation (Tulloch et al., 2011).

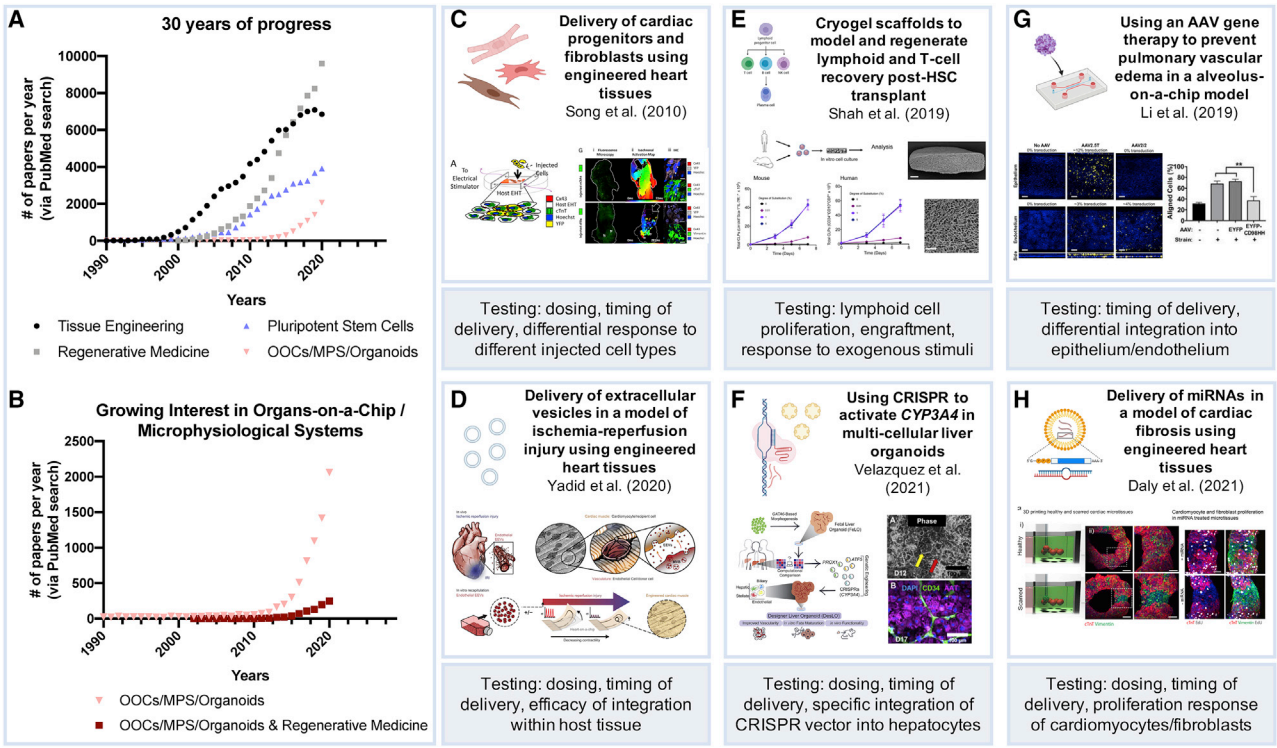

Figure 3. OOCs and related technologies for testing regenerative therapies *in vitro*

(A and B) Growth of the field (NIH PubMed database).

(C) Engineered cardiac models for studying engraftment of injected cardiomyocyte progenitors, testing timing of delivery and efficacy of different cell types on cardiac muscle function (Song et al., 2010); reproduced with permission, National Academy of Sciences.

(D) Extracellular vesicles (EVs) for hypoxia-induced myocardial infarction repair (Yadid et al., 2020); reproduced with permission, American Association for the Advancement of Science (AAAS)

(E) Lymphoid progenitor expansion *in vitro* can mimic testing cryogel scaffolds for hematopoietic reconstitution *in vivo* (Shah et al., 2019); reproduced with permission, Springer Nature Limited.

(F) Testing efficacy of CRISPR/Cas9 vector systems in liver organoids (Velazquez et al., 2021); reproduced with permission, Elsevier.

(G) Validating cell-specific integration of adeno-associated viruses within an alveolus-on-a-chip to mitigate pulmonary edema (Li et al., 2019); reproduced under the terms of the Creative Commons License.

(H) Elucidating the effects of specific miRNAs on cardiac muscle repair (Daly et al., 2021); reproduced under the terms of the Creative Commons License.

Models of cardiac muscle

Heart physiology is inherently different between animals and humans, limiting the capability of animal models—especially rodents—to faithfully recapitulate clinical outcome and serve as tools for mechanistic studies. Monolayer cultures of iPSC-CMs provide a simple way to study some aspects of cardiac biology. Co-culture of iPSC-CMs with other cell types such as MSCs, fibroblasts, and epicardial cells were shown to improve the iPSC-CM phenotype. However, the cell maturity remained far below that of the adult human cardiomyocytes (Yoshida et al., 2018; Zhang et al., 2019). While 2D cultures enable parallel studies of various cell types of the heart *in vitro* at high throughput (Sharma et al., 2017), 3D models are required for advanced maturation and physiological relevance. Aggregation of iPSCs into 3D cardiac microspheres have been reported to predict drug toxicity at high throughput and model cardiac disease (Giacomelli et al., 2020; Hoang et al., 2018; Mills et al., 2017, 2019).

Using lessons learned from regenerative medicine, 3D models of the heart are engineered to generate miniature functional units recapitulating different aspects of the cardiac muscle in a simplified manner, often not requiring complex vascular networks that limit large-scale regenerative efforts. Emphasis is placed on generating simple and robust models, guiding tissue anisotropy and electrical coupling to promote tissue maturation, and developing platforms that will enable real-time measurements and capture the interactions of the heart with other organ models (Figure 4).

In one of the early studies in the field, Kit Parker's group demonstrated the use of thin film-based cantilevers to support generation of anisotropic cardiac sheets in a "heart-on-a-chip" platform (Grosberg et al., 2011). However, while this platform provides an improved model of heart physiology compared to 2D cell cultures, it is not scalable to 3D. To promote the assembly of functional 3D tissue models, several groups have utilized anchoring pillars, posts, and wires to stretch cardiac tissues in order to provide mechanical loading (Boudou et al., 2012; Ronaldson-Bouchard et al., 2018; Schaaf et al., 2011; Tiburcy et al., 2017). Mathur and colleagues fabricated a microfluidic device consisting of anisotropic cardiac tissues and controlled perfusion to mimic diffusion transport to the cardiac muscle (Mathur et al., 2015). Nunes et al. demonstrated the use of a "Biowire" model to electrically stimulate and pace cardiomyocytes cultured on a wire, showing increased ultrastructural organization, calcium handling, and conduction velocity (Nunes et al.,

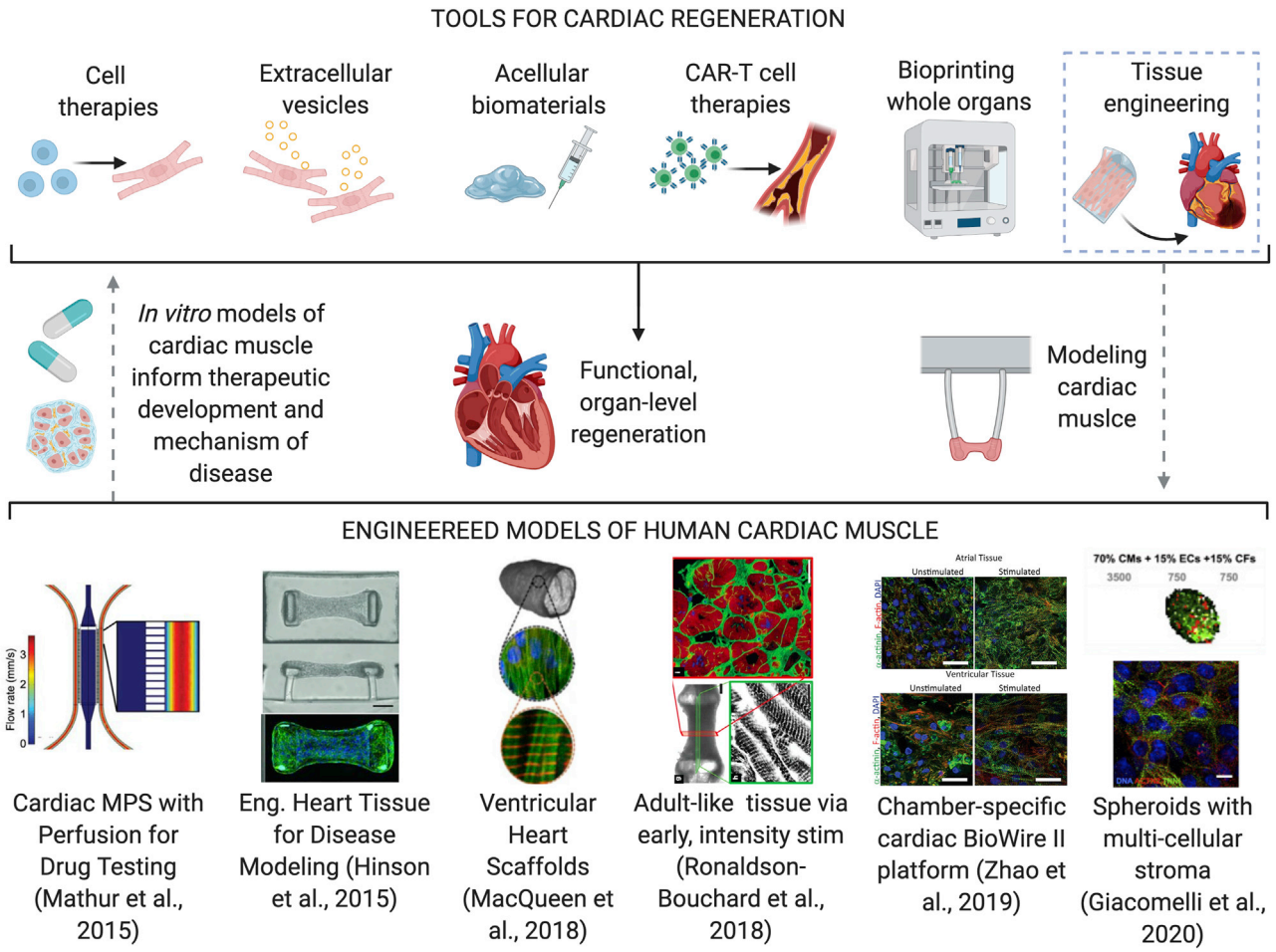

Figure 4. Engineered cardiac models for testing cardiac regeneration therapies

Top: dotted lines demonstrate the envisioned pathway of using bioengineered tools to make mimetic models of cardiac muscle and use such models to inform new therapeutic developments. Bottom: representative examples of recent cardiac muscle models: (Mathur et al., 2015), reproduced under the terms of the Creative Commons License; (Hinson et al., 2015), AAAS; (MacQueen et al., 2018), reproduced with permission, Springer Nature Limited; (Ronaldson-Bouchard et al., 2018), reproduced with permission, Springer Nature Limited; (Zhao et al., 2019a), reproduced with permission, Elsevier; (Giacomelli et al., 2020), reproduced under the terms of the Creative Commons License.

2013). Zhang and Khademhosseini endothelialized engineered cardiac muscle by bioprinting and harnessed this model in studies of vascular changes in myocardium (Zhang et al., 2016b). Several teams have subsequently coupled cardiac tissues with biosensors and advanced imaging techniques to dynamically monitor functional responses (Bhise et al., 2014; Feiner et al., 2016; Sidorov et al., 2017; Zhang et al., 2017; Zhao et al., 2019b).

Cardiac toxicity, which is a major cause of drug withdrawal, motivated the need for reliable human tissue models for drug screening. Long-term cultures (>4 weeks) were conducted by several groups to study disease progression and drug responses using gene-edited iPSC lines (Ma et al., 2018; Mathur et al., 2015).

By using iPSCs, a multitude of genetic cardiac disorders, including the dilated cardiomyopathy, *PRKAG2* cardiomyopathy, Barth syndrome, and Timothy syndrome, were recapitulated *in vitro* for mechanistic, developmental, and therapeutic studies (Hinson et al., 2016; Sun et al., 2012; Yazawa et al., 2011). For example, in a study investigating the significance of titin-based mutations in iPSC-derived cardiomyocytes, their active role in relaying sarcomere deterioration was identified as a cause for dilated cardiomyopathy (Hinson et al., 2015). A different study in a pillar-based model demonstrated the importance of full-length titin and MHC-β in sarcomerogenesis (Chopra et al., 2018). Cardiac fibrosis models have been developed by several groups in recent years, highlighting the opportunities to study mechanisms of cardiac disease progression and remodeling and evaluate the utility of antifibrotic drugs (Mastikhina et al., 2020; Nunes et al., 2013; van Spreeuwel et al., 2017). In studies integrating the cardiac and liver tissues, several groups have demonstrated drug metabolism along with known cardiotoxic side effects (McAleer et al., 2019; Pires de Mello et al., 2020).

To generate a cardiac model with adult-like characteristics, numerous groups have promoted tissue and cell maturation by long-term culture; addition of supporting cells; new media formulations; and mechanical, electrical, and hydrodynamic stimulation (Eng et al., 2016; Feyen et al., 2020; Jackman et al., 2016;

Nunes et al., 2013; Shadrin et al., 2017; Tiburcy et al., 2017; Zhao et al., 2019a). To build upon some of this work, our lab has investigated maturation of iPSC-derived cardiomyocytes *in vitro* (Ronaldson-Bouchard et al., 2018) by culturing early stage iPSC-cardiomyocytes (immediately after the initiation of spontaneous contractions) in fibrin hydrogels anchored between two pillars to support auxotonic contractions. The resulting cell-hydrogel constructs were exposed to an intensity training regimen at the gradually increasing electrical stimulation frequency starting from 2 Hz to 6 Hz over three weeks of culture, and then stabilized at 2 Hz for an additional week in culture. This regime of stimulation markedly improved the molecular, structural, and functional tissue properties toward more mature phenotype. Other groups also reported additional methodologies for maturing iPSC-derived cardiomyocytes more recently (Feyen et al., 2020; Giacomelli et al., 2020), derivation of ventricular-like and atrial-like cardiac tissues (MacQueen et al., 2018; Patel et al., 2016; Zhao et al., 2019a), and producing sinoatrial node pacemaker cells from iPSCs (Goldfracht et al., 2020; Protze et al., 2017). The directed cell differentiation is especially important for the cardiac muscle, as some drugs and diseases are known to affect different subpopulations of cardiac cells or regions of the cardiac muscle. For example, specific drugs target the atrial chambers and lead to atrial fibrillation, while some diseases, such as systemic lupus erythematosus, are known to affect mainly the left ventricle (Wijetunga and Rockson, 2002).

The choice of a specific cardiac model system should be based on the biological question at hand. 2D and microsphere co-cultures of human cells are highly suitable for high throughput studies. While microspheres are more physiologically relevant than 2D cultures (Ravenscroft et al., 2016), both systems do not capture the 3D complexity of cardiac tissues and do not incorporate architectural elements (i.e., stroma, ECM). Microfluidic channels in heart-on-a-chip models enable modeling of drug transport (Mathur et al., 2015); however, these systems are primarily fabricated out of polydimethylsiloxane (PDMS), which non-selectively absorbs hydrophobic molecules including oxygen and many drugs (Bhatia and Ingber, 2014). Engineered cardiac tissues in OOC platforms can capture more closely the complexity of the heart, and their enhanced maturity is especially important to predict physiological responses, such as calcium handling and force generation (Ronaldson-Bouchard et al., 2018; Zhao et al., 2019a).

Cardiac repair therapies

Cell delivery studies began in the early 1990s with the goal to restore cardiac function post-myocardial infarction and treat other cardiovascular disorders such as cardiomyopathy and congenital heart disease (Reinlib and Field, 2000). Many cell types have been used, including skeletal muscle cells, hematopoietic cells, endothelial cells, MSCs, epicardial cells, and the ESC- and iPSC-derived cardiomyocytes, with the anticipation that each type of cell delivery may benefit the damaged myocardium in a certain way (Ishida et al., 2019; Orlic et al., 2001; Yanamandala et al., 2017; Ye et al., 2014). However, the mechanistic basis for cell therapy still remains unclear. Successful delivery of ESC-derived cardiomyocytes has been achieved in non-human primates by the Murry group, with indications of success that included remuscularization of the heart and improved left ventricle function (Chong et al., 2014; Liu et al., 2018b). However, arrhythmias and low retention of cells have appeared in late preclinical trials. Emerging technologies, such as gene therapies to overexpress cell cycle activator *CCND2* in iPSC-derived cardiomyocytes, has proven to increase remuscularization and angiogenesis of injured cardiac muscle once implanted *in vivo* (Zhu et al., 2018).

Engineered cardiac tissues can overcome the challenge of poor cell engraftment. One of the first studies that designed engineered cardiac tissue for transplantation in animal models was reported by Leor and Cohen in 2000, leading the way in assessing tissue engraftment and efficacy of repair of the damaged cardiac muscle (Leor et al., 2000). Similar to the *in vitro* models of the heart, many groups have used the recent success in iPSC-derived cardiomyocytes to engineer cardiac patches for regeneration (Bejleri et al., 2018; Jackman et al., 2018; Tiburcy et al., 2017; Zhang et al., 2018). Menasché and colleagues reported the first clinical study of ESC-derived cardiac progenitor cells in a 68-year-old patient, followed by a larger clinical trial in 6 patients with severe ischemic left ventricular dysfunction, suggesting the safety and efficacy of using pluripotent stem cells (Menasché et al., 2015, 2018). While these clinical studies represent a major breakthrough for the field, critical challenges, such as patch vascularization and immune rejection, still remain and should be addressed to ensure clinical translation (Edri et al., 2019; Fleischer et al., 2020).

Over the past five years, many groups have been attempting to incorporate vasculature into engineered cardiac tissues to ensure the immediate supply of oxygen and nutrients to the implanted cells (Gao et al., 2017; Ye et al., 2014). Zhang and Radisic demonstrated the development of an ''AngioChip'' system for engineered vasculature in highly dense, millimeter-thick tissues that enables direct anastomosis to host for immediate perfusion (Zhang et al., 2016a). Future developments in this space will rely on vascularizing tissues and on their integration with host vasculature, using different bioengineering approaches, including 3D bioprinting (Lee et al., 2019; Noor et al., 2019; Skylar-Scott et al., 2019).

Other groups have studied the role of cell secreted microRNAs (miRNAs) in cardiac repair (Eulalio et al., 2012; Shao et al., 2017). Namely, the delivery of the secretome and extracellular cargo of regenerative cells has been indicated as a potential mechanism for alleviating cardiac damage following myocardial infarction (Liu et al., 2021). EVs containing miRNAs that are secreted by human ESCs, iPSCs, ECs, MSCs, and iPSC-derived CMs have all been implicated in regeneration of the heart after exogenous delivery (Adamiak et al., 2018; Akbar et al., 2017; An et al., 2017; Bian et al., 2014; Khan et al., 2015). Recently, our group reported that extracellular vesicles collected from human iPSC-derived cardiomyocytes were superior to those collected from iPSCs in promoting a pro-regenerative phenotype in infarcted hearts, suggesting that the cardiogenic phenotype of cells used to produce EVs is important for treating infarcted hearts (Liu et al., 2018a). Another group has shown beneficial effects of EVs secreted by vasculogenic cells in treating infarcted heart models (Yadid et al., 2020). However, the engraftment of EVs, their off-target effects, and mechanisms of action (prevention or treatment of cardiac fibrosis) still remain as unknowns in translational cardiac repair.

Harnessing emerging bioengineering tools for cardiac regeneration

A new wave of bioengineering approaches to cardiac regeneration have appeared following advances in other fields. Over the past decade, gene editing technologies have enabled therapies based on manipulating diseased cells *in vivo*. Although much more work needs to be done, some groups have attempted functionalizing viral carriers into engineered scaffolds for controlled viral vector release (Gu et al., 2017). Qian and Srivastava reported in 2012 the selective reprogramming of cardiac fibroblasts into cardiomyocyte-like cells *in situ* (Qian et al., 2012), using transcriptional expression of Gata4, Mef2c, and Tbx5, though challenges remain in efficacy and functional maintenance long-term (Tani et al., 2018). For example, by understanding the role of ECM-derived proteins in development, Bassat and Tzahor uncovered the role of agrin in promoting cardiomyocyte proliferation and muscle regeneration post-MI, demonstrating the importance of the microenvironmental cues in promoting *in vivo* regeneration (Bassat et al., 2017). Recently, Aghajanian and Epstein demonstrated engineering of CD8+ T cells to target cardiac fibroblasts after infarction-induced fibrosis in mice (Aghajanian et al., 2019). This study, although at a proof-of-concept level, showed that targeting fibroblast activation protein via chimeric antigen receptors (CAR) on T cells could advance immunotherapy past their recent applications in treating cancer.

Outlook

Stem cell and cardiac tissue engineering therapies are currently not routinely used in the clinic (Menasché et al., 2018). Although these therapies have demonstrated capability to prevent or reduce heart injury, the precise mechanisms remain largely unknown (Curfman, 2019; Vagnozzi et al., 2020). Gaining a better mechanistic understanding of these processes can greatly advance the field toward clinical translation.

Recent developments in modeling the healthy and injured myocardium using OOCs (Giacomelli et al., 2020; Mastikhina et al., 2020; Ronaldson-Bouchard et al., 2018; Wang et al., 2019; Zhao et al., 2019a) suggest that these *in vitro* systems could emulate some aspects of injury and study regeneration therapies, in conjunction with animal models. The 2010 collaborative effort from the Zandstra and Radisic groups shows one of the first forays into the concept of using an engineered model to study regenerative cell therapy (Figure 3C) (Song et al., 2010). In MI, engineered models of ischemia-reperfusion injury can help inform the administration of new drugs and biologics, by mimicking what is seen in the clinic (hours to days after heart attack) (Chen and Vunjak-Novakovic, 2019). Recent applications using exosome-based therapies in engineered models of MI have enabled the optimization of exosome delivery regimens necessary to decrease acute fibrosis (Wagner et al., 2020; Yadid et al., 2020). Models of disease are constantly improving, with cardiac organoids being used to model important hallmarks of MI—including metabolic shifts, fibrosis, and changes to functional calcium handling (Richards et al., 2020). In a recent study of iPSC-derived cardiac microtissues by the Mummery group, cardiac stromal cells, endothelial cells and fibroblasts, were all found to be contributing to cardiomyocyte maturation and reducing progression of cardiac injury (Giacomelli et al., 2020). Of particular note, the specificity of cell types from cardiovascular lineages, like in cardiac fibroblasts as compared to dermal fibroblasts, was important for better sarcomere formation *in vitro*. Understanding how stromal to parenchymal cell ratios in engineered models may parallel *in vivo* organ makeup is an ongoing effort in the field. In studying early gestational development, Drakhlis et al. recently demonstrated that human heart-forming organoids could model the structural complexity of early heart and foregut development, with potential to model genetic mutations causing cardiac malformation, another area where OOCs can serve as a template for regenerative interventions in congenital disease (Drakhlis et al., 2021).

OOC systems could also help identify novel mechanisms of action for regenerative therapies, while decoupling the effects of multiple factors, an important aspect that is difficult to achieve in animal models (Figure 4). Some of the outstanding questions of interest include:

- *How are regenerative treatments affecting tissue function over time (e.g., by attenuating myofibroblast activation and fibrosis, promoting cardiomyocyte survival, inducing vascularization)?*
- *What are the underlying mechanisms of repair in different cell types, and what is the route of action (paracrine factors, direct contact)?*
- *What is the time frame for therapy? Are certain therapies more advantageous during the inflammatory phase, while others are beneficial during inflammatory resolution?*
- *What role do immune cells play in the progression of MI and regeneration?*
- *What are the changes that occur in the cardiac ECM following regenerative therapies (e.g., stiffening, degradation, deposition of specific proteins)?*

Overall, modeling of cardiac injury and regeneration using OOCs would help advance future work and clinical translation.

Respiratory system: Modeling alveoli to advance lung regeneration

The lung airway is made of a dense network of branching units, from the proximal bifurcation of the mainstem bronchi to the most distal oxygen-exchanging alveoli, that are intertwined with the equally complex vascular network (Basil et al., 2020), resulting in gas exchange surface of nearly 200 m^2. With a highly complex structure, about 23 airway generations, over 40 cell types, and 150 different native ECM components, recapitulating lung physiology and functionality for disease modeling or regeneration remains a major challenge in the field. Some of the first reports in the OOC field were from the Ingber group, where a model of the airway epithelium, with functional oxygen exchange and barrier protection metrics, was developed in 2010 (Huh et al., 2010). Many groups since then have recapitulated one or more of the functional metrics for bioengineered airway and used these model systems to study systemic diseases. To date, only a few groups have attempted large-scale, clinical-grade techniques for restoring lung function. As the lung is an extremely complex organ, several groups have relied on advances in bioengineering technologies that restore functional lungs starting from damaged or diseased donor lungs (Basil et al., 2020).

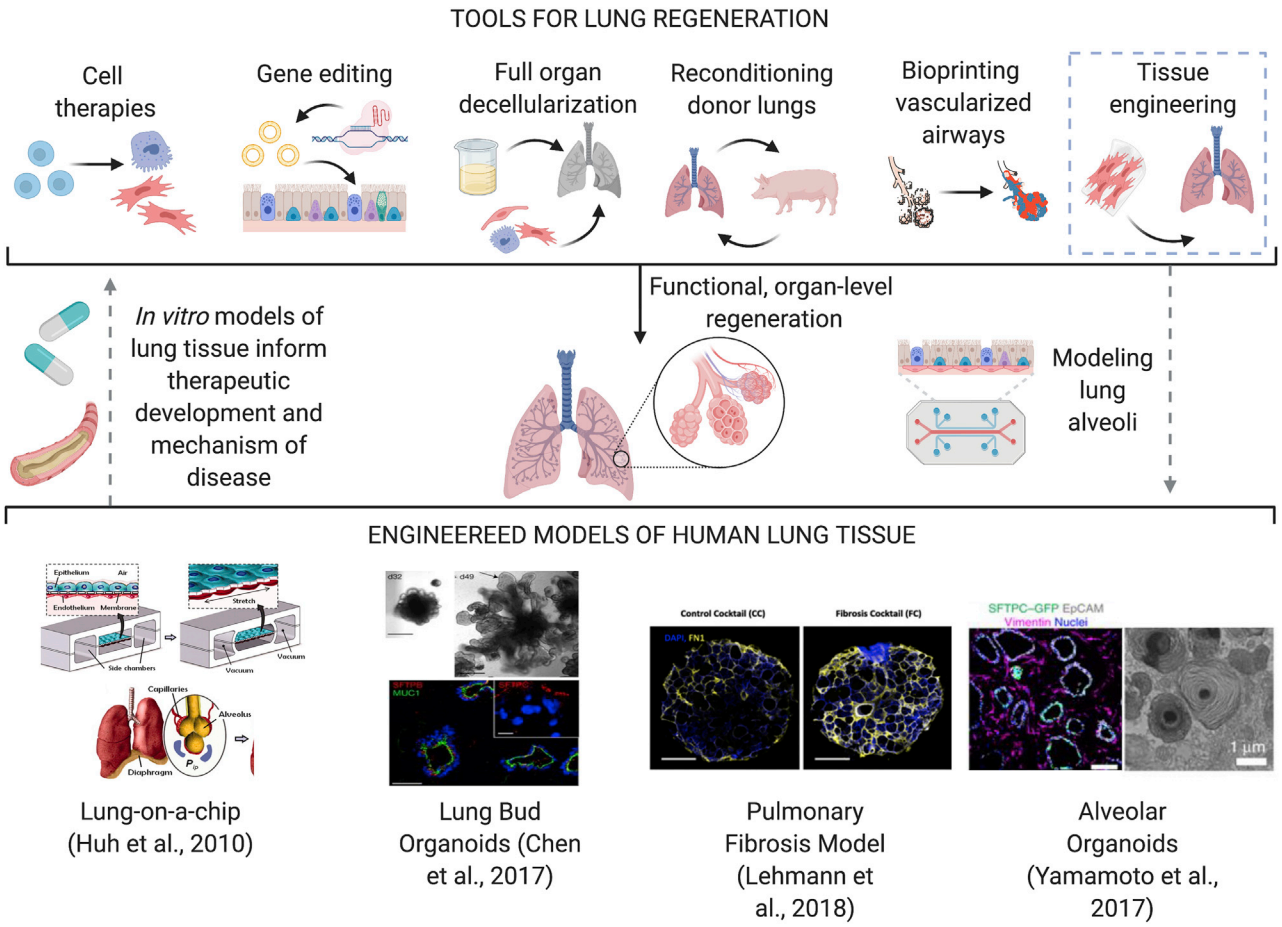

Figure 5. Engineered alveolar models for testing novel therapies for macro-scale lung regeneration

Top: Dotted lines demonstrate the envisioned pathway of using bioengineered tools to make mimetic models of the lung and use such models to inform new and refined therapeutic development. Bottom: Representative examples of lung tissue models: (Huh et al., 2010), copyright 2010, AAAS; (Chen et al., 2017), reproduced with permission, Springer Nature Limited; (Lehmann et al., 2018), Reproduced under the terms of the Creative Commons License; (Yamamoto et al., 2017), reproduced with permission, Springer Nature Limited.

Models of lung physiology

Huh and Ingber demonstrated in 2010 the ability to create a perfusable alveolar epithelial-endothelial barrier *in vitro* (Figure 5). This simple *in vitro* model was shown to recapitulate the organ-level functions of the lung, such as responses to bacterial infection, inflammation, and mechanical behavior simulating breathing (Huh et al., 2010). Since this seminal work, Ingber's group has demonstrated the potential to use the "lung-on-a-chip" to study drug-induced pulmonary edema, intravascular thrombosis, and non-small-cell lung carcinoma (Hassell et al., 2017; Huh et al., 2012; Jain et al., 2016, 2018). These devices were benchmarked against clinically observed drug responses. One of the first and highly successful commercial ventures in the OOC space, the company Emulate, Inc., has emerged from this early work. Prior to this, many groups had studied primary human lung epithelium using 3D air-liquid-interface (ALI) models that were able to recapitulate mucus secretion, cilia formation, and ECM remodeling (Pezzulo et al., 2011; Yamaya et al., 1992). Raredon and Niklason demonstrated that the use of a rolling bioreactor to establish a constantly dynamic ALI culture was important for increasing the number of ciliated cells, mucus secretion, and barrier function in differentiating epithelial cells (Raredon et al., 2014).

Starting from the donor lungs, many groups have established engineered systems to study the lung *ex vivo* for drug testing and disease progression in organotypic cultures. Königshoff and colleagues demonstrated the effective culture of primary human lung slices for up to 21 days, using samples from both healthy donors and patients with idiopathic pulmonary fibrosis (IPF), allowing enough time to assess efficacy of potential therapeutics (Alsafadi et al., 2017; Bailey et al., 2020; Lehmann et al., 2018). This approach takes advantage of using primary tissue samples to preserve alveolar architecture, as complex lung structures are often neglected in *in vitro* lung models.

To establish a source of human cells for lung regeneration, Snoeck and colleagues developed a protocol for highly efficient differentiation of lung progenitors and airway epithelial cells from pluripotent stem cell sources. In 2014, their group was able to generate basal, goblet, Clara, ciliated, type I, and type II alveolar epithelial cells from ESC and iPSC sources, with effective functional metrics and progenitor proliferation *in vitro* (Huang et al., 2014, 2015). These studies were instrumental

for providing a milieu for human, patient-specific drug testing and disease modeling *in vitro* (Snoeck, 2015). The group developed a 3D lung bud organoid model, capable of effectively producing mesodermal and pulmonary endodermal populations (Chen et al., 2017). These organoids had mobile cilia, secreted airway mucus, were maintained *in vitro* for over 170 days, engrafted with host vasculature, and formed tubules following transplantation in immunodeficient mice. In addition, it was demonstrated that lung organoids were effective for disease modeling of viral infection, fibrosis, and genetic diseases of the lung (Porotto et al., 2019; Strikoudis et al., 2019; Yamamoto et al., 2017).

Organoids can be used for high throughput studies of disease modeling and drug screening. However, these models often lack essential cellular components, such as immune and vascular progenitors, which play major roles in homeostasis and response to injury. Like for most iPSC-derived models, maturation is a major concern, reaching only fetal-like status in even long-term (weeks to months) culture. Establishing gas exchange in lung organoids remains a challenge, as lack of integrated vasculature prevents this essential lung function (Tian et al., 2020). Further, lack of tissue-specific ECM overlooks the scarring that often progresses in diseases like IPF. 3D tissue ALI cultures, including those within microfluidic chips, incorporate perfusion flow to mimic the endothelial-epithelial barrier, but only at medium-to-low throughput, which limits their widespread use. Validation metrics include barrier function/permeability, multipotent epithelial cell maintenance and differentiation, and ability to respond to acute injury. As with any organ system, the balance between throughput and complexity is essential, with the question of interest often informing the choice of human model system.

Lung regeneration

Approaches to regenerating whole lungs have ranged from repopulating decellularized scaffolds with lung epithelial cells to clinical devices for normothermic *ex vivo* lung perfusion (EVLP), which aim to maintain lungs outside the body prior to transplant. The range of techniques have crossed the boundaries of repairing damaged donor lungs from the "top down" (in the case of EVLP) to creating new organs or tissues from the "bottom up" (in the case of tissue engineering) (Pinezich and Vunjak-Novakovic, 2019). In newer approaches, cell and gene delivery methods are aiming to target specific replacement of damaged epithelium without invasive transplantation (Dorrello and Vunjak-Novakovic, 2020).

In the early 2000s, bioengineers developed methodologies to remove the epithelial and endothelial cells from the lung matrix while maintaining the native lung architecture and ECM components (Gilbert et al., 2006). Ott et al. developed a protocol for bioreactor-based perfusion-decellularization of the heart that maintained perfusable vascular channels and then recellularized the heart matrix with cardiomyocytes and endothelial cells over four weeks of bioreactor culture (Ott et al., 2008). In Ott and Vacanti's follow up work in the lung, and in parallel studies by Peterson and Niklason, it was demonstrated that entire lungs can be decellularized, with preservation of intact hierarchical branching, and then recellularized by infusing epithelial and endothelial cells (Ott et al., 2010; Petersen et al., 2010). These engineered lungs were perfused through the vascular channels and ventilated through the airway at physiological conditions. Following implantation, the lungs survived for only a short period of time (several hours), largely due to the lack of functional vasculature. These studies demonstrated both the potential of this approach and the need to improve and extend the lung functionality. These seminal papers enabled the field to use decellularization of whole organs (heart, lungs, etc.), to obtain fully biological scaffolds for organ engineering. To maintain blood supply to the lung, Dorrello et al. demonstrated selective de-epithelization of the lung with the maintenance of intact vasculature and branching hierarchy (Dorrello et al., 2017). After re-seeding de-epithelialized lungs, human pulmonary epithelial cells were shown to engraft and expand, suggesting potential application in epithelial disease treatment and transplantation of bioengineered human lungs. Other targeted delivery methods have been explored for controlled delivery of cells, drugs, and reagents to restricted regions of the lungs, for spatial reorganization of damaged lung regions *in vivo* (Kim et al., 2015, 2017).

In the context of donor organ recovery, EVLP bioreactors have been widely explored and notably, translated into clinical use. Keshavjee and colleagues demonstrated the extension of normothermic EVLP to up to 12 h (Cypel et al., 2008). In tandem, the group tested anti-inflammatory cytokine IL-10 on *ex vivo* perfused lungs to avoid graft dysfunction and retain gas exchange functionality of alveoli prior to transplantation (Cypel et al., 2009). Our group has extended the duration of extracorporeal support of donor lungs on ventilation and cross-circulation with a recipient from 6–12 h to as long as 100 h (Hozain et al., 2020b; Huang et al., 2014). Using a host swine model as a bioreactor, donor lungs rejected for transplant were recovered and maintained *ex vivo* (Hozain et al., 2020b). These techniques enable a number of future bioengineering therapies in donor lungs, including cell and gene delivery, and can be extended to severely damaged lungs after gastric aspiration injury (Guenthart et al., 2019; Kim et al., 2015). The efficacy of cell therapy of the lung is yet to be proven in humans, though the technology holds immense potential for treatment of diseases that affect the epithelium (Ghadiri et al., 2016). In a more recent study by our group, we have extended the use of cross-circulation to regeneration of human donor lungs to the levels needed for transplantation, in the first study of its kind (Hozain et al., 2020a). Notably, donor lung vasculature remained intact for the duration of the study, with stable blood-gas barriers and no signs of angiopathy. In many cases of whole organ lung repair, maintaining stable and functional vasculature remains a major challenge, especially in regards to anastomosis to host tissue once implanted. Throughout the study, Hozain et al. assessed the metabolic and immunogenic reactions between the swine host and the human lungs. Using *in vitro* OOC models could be useful in these cases to test the xenogenic contributions of the host on the human tissue and to assess any adverse reactions prior to transplantation.

Harnessing bioengineering tools for lung regeneration

Gene therapies targeting epithelial cells would allow for targeted correction of single-gene defects in inherited lung disorders. *In vivo* correction of the *CFTR* gene has been explored for treating cystic fibrosis (Griesenbach et al., 2016). Firth et al. demonstrated the ability to use CRISPR to correct iPSCs derived from cystic fibrosis patients with a deletion of F508 in the *CFTR* gene.

After correcting this common mutation, they were able to generate airway epithelial cells from the corrected iPSCs, suggesting the potential for generating healthy, patient-matched lung epithelia for genetic lung diseases (Firth et al., 2015). Osman et al. recently developed polyethylene-glycol (PEG)-coated nanoparticles for increasing the efficacy of transfection in mucus-coated membranes, like the lung epithelia, enabling better *in situ* options for gene therapies (Osman et al., 2018). With major developments in high-resolution 3D biomanufacturing techniques, many groups, including the Feinberg and Lewis teams, have independently pursued the integration of perfusable vasculature into bioprinting of human organs—in the heart and kidney, as examples (Homan et al., 2019; Lee et al., 2019; Skylar-Scott et al., 2019). Of note, the Stevens and Miller groups recently reported the formation of highly complex, vascularized alveolar hydrogel structures using stereolithography (Grigoryan et al., 2019). Capable of ventilation and oxygenation *in vitro*, red blood cells flowed in and out of the alveolar structures, showing potential functional benefit of these engineered constructs in recapitulating critical gas-exchange properties of the lungs. Advances in biomanufacturing are helping target one of the most difficult challenges in lung regeneration, whereas scalable, multi-lobular lungs continue to remain a long-range goal for the bioprinting field.

Outlook

Advances in lung regeneration *in situ* and *ex vivo* show promise for restoring lung function in pre-clinical models. With advances in gene editing and bioengineering cell therapies, there is a large space for collaborative efforts to target genetic lung diseases, such as in cystic fibrosis, and restore functional lung epithelium. Many groups are developing strategies for studying genetic and acute lung diseases *in vitro*, using primary tissue samples for high biological fidelity and patient-specific models. In such diseased microenvironments, the roles of specific cellular components (epithelial cells, macrophages, and fibroblasts) and extracellular remodeling processes (collagen deposition, and MMP secretion) can be studied using OOC models to facilitate therapeutic development (Figure 5) (Castellani et al., 2018).

Models of the lung, including those that recapitulate alveolar gas exchange, can serve as tools for studying the effects of new therapeutics targeting epithelia. For example, these models could help (1) investigate how the microbiomes of healthy and diseased lungs impact their ability to respond to injury and therapeutics, (2) how the innate and adaptive immune components remodel the alveolar microenvironments during injury and repair, and (3) what is the role of therapeutic MSCs in the epithelial-to-mesenchymal transition (Basil et al., 2020). With an organ as complex as the lung, it is essential to understand and decouple the roles of specific cell types, extracellular matrix, and environmental contaminants in the context of injury or disease. Lung progenitor cell populations are crucial for the natural regenerative processes, and replacing damaged epithelium comes with questions about maintaining the structure and function of this mechanically dependent organ. For example, in cases of targeted replacement of epithelial cells, an engineered lung model can help determine whether damaged epithelial cells can be replaced to drive lung remodeling while the innate vasculature remains intact. Another interesting question is whether CRISPR-corrected iPSC-derived epithelial cells from CF patients are able to engraft and replace damaged mucosal epithelium (Firth et al., 2015). In most cases, precise alveolar architecture may be required for reliable *in vitro* testing, similar to the model described by the Stevens and Miller groups (Grigoryan et al., 2019).

Parallel experiments in large animal models that are close to human lung size and physiological function and in patient-specific OOC models would help drive the development of new treatment modalities, speed up the route to translation, and provide personalized approaches to lung regeneration. OOC models could help mitigate lung injuries due to ischemia and gastric aspiration by serving as a test-bed for studying candidate therapies (Guenthart et al., 2019). With the emergence of cross-circulation system to regenerate damaged lungs, there is a need to better understand xenogeneic relationships between the porcine host and human lung, with both a presence and absence of immune components, which may exacerbate damage (Guenthart et al., 2019; Hozain et al., 2020a, 2020b). Although there is potential to synergize OOC models with regenerative lung therapeutics, (Figure 5) there are many outstanding questions:

- *With over 40 types of cells with many different functions in the lung, how can we elucidate the effects of different treatment modalities on specific groups of cells?*
- *What are the effects of regenerative therapies on the stromal and endothelial cell populations of the lung?*
- *How do selective cell replacement therapies alter the alveolar microenvironment?*
- *What is the time window for therapy during acute and chronic lung injury?*
- *What are the immunological effects of autologous versus allogenic cell therapies? And what role do lung-resident immune cell populations play in it?*

In the age of the SARS-CoV-2 (COVID-19) pandemic, engineered model systems are being repurposed for mechanistic studies of viral infection and therapeutic discovery and validation in human tissue platforms (Lamers et al., 2020; Monteil et al., 2020). For the lung, where the majority of fatalities result from acute respiratory distress syndrome (ARDS), epithelial organoids or lung OOC systems are proving to be excellent tools for disease modeling and therapeutic discovery (Abo et al., 2020; Mulay et al., 2020; Si et al., 2021). With the field of lung regeneration at its early stages, there is immense potential for using OOC models for mechanistic and proof-of-concept studies of bioengineered human lung tissues.

Blood and immune system: Modeling the bone marrow for regenerative hematopoiesis

In hematopoiesis, it is widely accepted that a single blood stem cell has potential to regenerate the entire blood and immune system. Commonly found in the bone marrow, hematopoietic stem and progenitor cells (HSPCs) are regulated by a number of microenvironmental cues that regulate their self-renewal and differentiation, with ability to respond to stress signals throughout the body during times of injury. The bone marrow niche is highly complex, often requiring a variety of stromal cellular components to allow for proliferation of HSCs and differentiation of

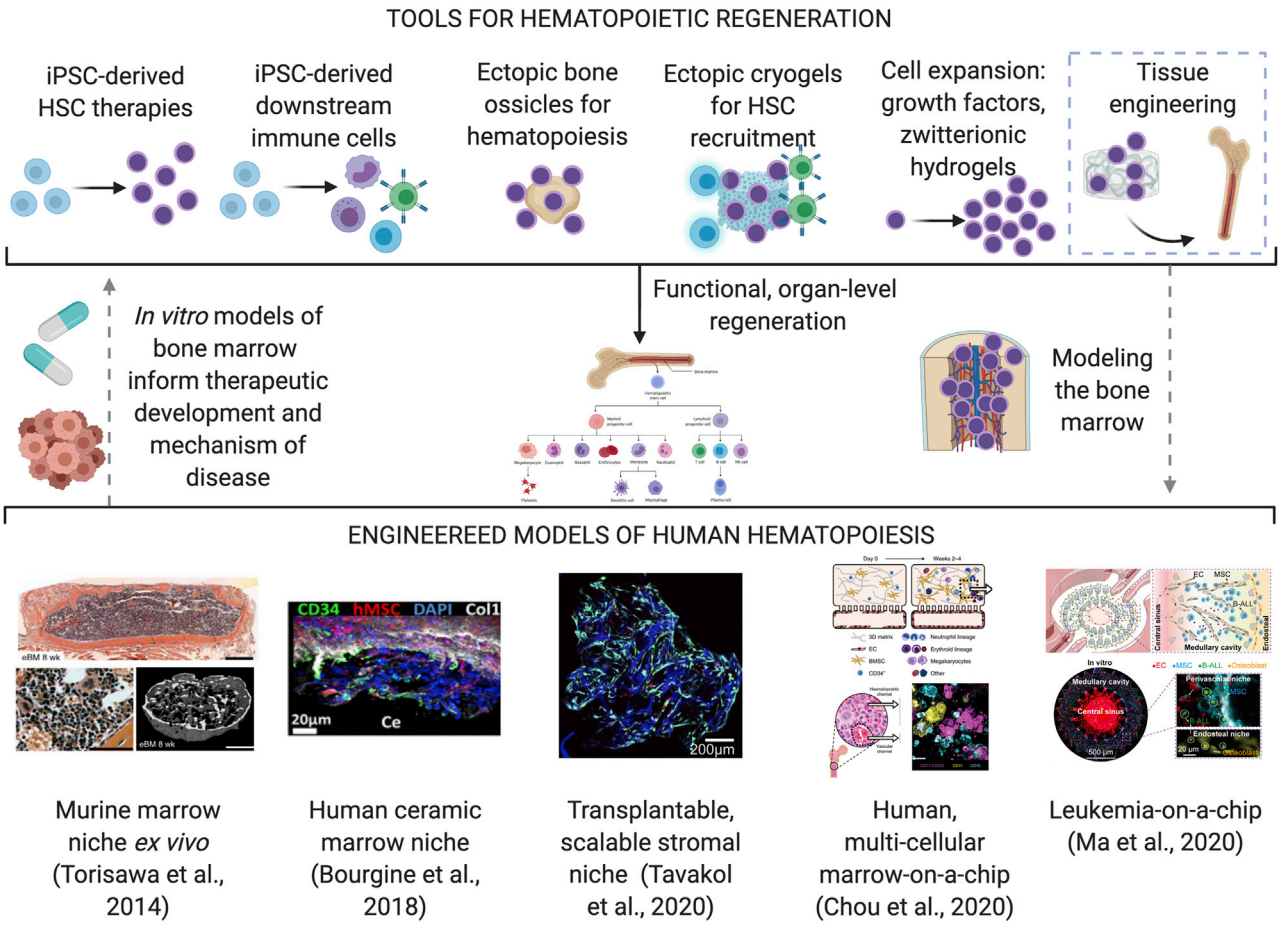

Figure 6. Engineered models of the bone marrow for testing hematopoietic regeneration

Top: Dotted lines demonstrate the envisioned pathway of using bioengineered tools to make mimetic models of the bone marrow and use such models to inform new and refined therapeutic development. Bottom: representative examples of hematopoietic models: (Torisawa et al., 2014), reproduced with permission, Springer Nature Limited; (Bourgine et al., 2018), reproduced with permission, copyright 2018, National Academy of Sciences; (Tavakol et al., 2020), reproduced with permission, copyright 2019, Elsevier; (Chou et al., 2020), reproduced with permission, Springer Nature Limited; (Ma et al., 2020), reproduced with permission, copyright 2020, AAAS.

progenitors into downstream blood and myeloid/lymphoid lineages (Morrison and Scadden, 2014).

In recent years, different cellular components, including endothelial cells, adipocytes, and osteoblasts, have all been shown to regulate hematopoiesis in both health and disease (Coutu et al., 2017; Kobayashi et al., 2010; Naveiras et al., 2009; Zhou et al., 2017). In treating hematological disorders and cancers of the blood, full-body irradiation and subsequent hematopoietic stem cell transplant had been the traditional options for reducing or curing symptoms of disease (Copelan, 2006), with more recent developments in small molecule and CAR-T therapies reaching the clinic in recent years (June et al., 2018). Early success with HSC transplantation (HSCT) in the 1950s has allowed for the first successful stem cell transplants for regenerative therapies still ongoing to this date; however, approximately 25% of these transplants fail to engraft, causing downstream complications for patients. In addition, immunological rejection, like graft-versus-host disease (GVHD), can emerge as complications to immunosuppressants. With the large number of blood disorders and the growing number of blood and immune cell therapies in pursuit, approaches to studying these treatments in human, *in vitro* models may lead to more mimetic efficacy and safety research, as well as studying the mechanism of cell delivery, engraftment, and survival in the host tissue context.

Models of the hematopoietic niche

The murine hematopoietic niche has long been studied for preventing the onset of blood cancers and age-related exhaustion of hematopoietic cells (Doulatov et al., 2012). In fact, much of our understanding of the human hematological disorders comes from mouse models that are still a gold standard in the field. Also, many engineered models to study *in vitro* diseases of the blood and marrow rely on using murine hematopoietic cells (Figure 6). The necessity of stroma in these models has been demonstrated already in the initial co-culture experiments leading the way into the *in vitro* platforms to study hematopoietic support (Bianco et al., 2008; Friedenstein et al., 1974; Majumdar et al., 2000; Walenda et al., 2010). Butler and Rafii were the first to show that cord blood-derived CD34+ HSPCs expanded significantly better in co-culture with endothelial cells than in monocultures (Butler et al., 2012).

Other early approaches, such as the use of mesenchymal stromal cell spheroids to expand HSCs *in vitro* were developed to aid in expansion and engraftment *in vivo* (Isern et al., 2013). In one of the first studies using ectopic extramedullary hematopoietic niches, or sites of hematopoiesis other than the host bone marrow, Tavassoli and Crosby showed in 1968 that hematopoiesis was established in subcutaneous rat tissues after transplantation of stromal cells. The use of ectopic sites has paved a way for a number of hematopoietic models (Tavassoli and Crosby, 1968). Studies of hematopoiesis were markedly enhanced by the development of human ectopic hematopoietic niches through scaffold-free transplantation of MSC-derived cartilage tissues and selective differentiation into osteogenic/hematopoietic lineages via developmentally inspired approaches, like endochondral ossification, subcutaneously in animals (Scotti et al., 2013). Xenotransplantation of humanized stromal "ossicles" in mice have been used in studies of leukemia, affording a useful model system to study healthy and leukemic cell engraftment in a system capable for easy biopsy accessibility from subcutaneous tissue (Reinisch et al., 2016).

In the first "bone marrow-on-a-chip" model, Torisawa and Ingber established in 2014 murine hematopoiesis by implanting a PDMS mold with bone-inducing materials subcutaneously, explanting the engineered tissue with recruited stromal and hematopoietic cells, and testing the metrics for radio- and cytotoxicity of the marrow (Torisawa et al., 2014). This work used murine cells with limited culture times and is yet to be extended to wide-scale application of human models. In 2018, the first fully human OOC model of hematopoiesis was developed by the Martin group, using MSC-derived osteoblasts and cord-blood HSPCs in a ceramic scaffold, to demonstrate hematopoietic support and response to injury *in vitro* (Bourgine et al., 2018). In a follow-up study, a multi-cellular, human model was developed using endothelial cells and MSCs within fibrin hydrogels to support cord blood-derived HSPCs (Chou et al., 2020). This study demonstrated the clinical utility of the marrow model to study Shwachman-Diamond syndrome, as well as various toxicities of hematopoietic cells in response to radiation or chemotherapeutic exposure. These human models, though at their early stages, have opened the door to studying diseases of blood stem progenitor cells, as well as off-target measures of marrow toxicity.

In modeling immune cell function *in vitro*, bioengineering approaches have been explored to study single cell types in response to various stimuli or disease states. Spiller and colleagues have shown the utility of macrophages in driving vascularization of tissue-engineered materials, elucidating the role of macrophage subtype differentiation in early wound healing (Graney et al., 2020; Spiller et al., 2014). Other groups have explored the *in vitro* formation of neutrophil extracellular traps in response to material stiffness, modeling the *in vivo* response to biomaterial implantation (Abaricia et al., 2021). For studying more complex adaptive immune responses *in vitro*, several groups have developed lymphoid organs for modeling B and T cell responses *in vitro*. In recent years, generation of a lymph node-on-a-chip device enabled studies of dendritic and T cell interactions, including priming T cell activation in response to varying shear stresses (Moura Rosa et al., 2016). Similarly, the feasibility of deriving B cell organoids for modeling the germinal center response in helping propagate and differentiate naive B cells was reported (Purwada et al., 2015). Possible inclusion of secondary immune organs has been discussed in several excellent reviews (Irimia and Wang, 2018; Kim et al., 2019).

Like all OOCs, benchmarking hematopoietic and immune models to human patho(physiology) remains a challenge, as murine responses have been studied more extensively than human models (Doulatov et al., 2012). Validation metrics includes the ability to maintain hematopoiesis over time with multipotent colony-forming potential, transcriptional similarity to adult BM-HSPCs, and cytokine-directed differentiation into multiple blood lineages. Capturing the complexities of the hematopoietic niche remains a futile challenge, as it is still not possible to study the entire hematopoietic landscape in one model system. Simple models of the BM can be incredibly important in studying basic immune function and myelopoiesis, often providing a native, self-repopulating niche of immune progenitors to respond to injury. However, the engineered models of the BM generally lack maturation of progenitors to distinct differentiated cells, especially to adaptive immune cells. Integrating models of the BM with secondary lymphoid organs may prove effective at mimicking immune responses after these models are benchmarked and validated against whole organism responses.

Regenerative hematopoiesis

HSC transplants have resulted in the survival and recovery of millions of patients over the past 50 years (Singh and McGuirk, 2016). However, there is still a need for therapeutic cells that can be expanded *in vitro* while retaining capacity to engraft *in vivo*. Although hematopoietic cells are phenotypically HSCs via antigen identification, not all of these cells are functionally supporting blood regeneration. *In vitro*, colony-forming-unit (CFU) assays are often used to identify the multipotent, colony-forming cells. *In vivo*, the gold-standard for HSC functionality is engraftment in a host marrow niche after primary and secondary transplantation. Therefore, it is opportune to use these assays to validate the efficacy of HSCs derived from different sources and expand these cells *in vitro*. Many efforts over the past two decades have focused on deriving HSCs from ESC and iPSCs, using transcription factor overexpression, isolation from teratomas, and growth factor delivery (Irion et al., 2010; Lengerke et al., 2009; Wang et al., 2005).

Many groups have identified the need to induce a vascular phenotype, starting with a hemogenic endothelial population as found during development, prior to hematopoietic commitment (Kennedy et al., 2012; Sandler et al., 2014; Sturgeon et al., 2014). Sugimura and Daley demonstrated in 2017 effective generation of engraftable, functional HSCs from human iPS and ES cells by differentiation into a hemogenic endothelium fate and subsequent expression of seven transcription factors (ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1, and SPI1) (Sugimura et al., 2017). These cells were able to produce myeloid, B, and T cells and engraft in primary and secondary mouse transplantation, with the efficacy shown in half of the experiments. This translational improvement further enables modeling of patient-specific blood disorders *in vitro* toward developing therapeutic applications for autologous HSCT (Doulatov et al., 2017; Georgomanoli and Papapetrou, 2019). Of note, mutations in iPSCs derived from acute myeloid leukemia patients are retained after

reprograming, with gene expression signatures and methylation changes reacquired once differentiated to hematopoietic cells (Chao et al., 2017; Kotini et al., 2017). More recently, Wang and Papapetrou demonstrated the gradual clonal evolution of leukemia from pre-malignant states (clonal hematopoiesis and myelodysplastic syndrome) to malignant acute myeloid leukemia using gene editing of iPSCs (Wang et al., 2021). As in native hematopoiesis, the stromal microenvironment from engineered models is still required to study chemotherapeutics, as leukemic blasts often alter their environment to propagate resistance and relapse (Rashidi and DiPersio, 2016). In response to these needs, *in vitro* models of leukemia are beginning to be explored (de la Puente et al., 2015; Ma et al., 2020).

Recently, Lis et al. demonstrated conversion of adult murine endothelial populations into HSCs through temporary overexpression of transcription factor-encoding genes Fosb, Gfi1, Runx1, and Spi1, potentially opening up a new source of cells that is independent of pluripotent cells (Barcia Durán et al., 2018; Lis et al., 2017). Though the promise that more directed differentiation from adult cell populations would alleviate the lengthy iPSC timeline and risk of inefficient pluripotent differentiation capacity, the potential for future malignant transformation of EC-derived HSCs has not been explored past 20 weeks *in vivo*. Importantly, the loss of epigenetic endothelial properties over longer periods of time was not confirmed and future studies are needed to address this important question. In addition to cell sourcing, maintaining and expanding HSCs *in vitro* has been a challenge. Different approaches have used different components to expand HSCs *in vitro* (high cytokine/growth factor culture media, hydrogels, co-cultures) in a way that maintains their functional capacity (Bello et al., 2018; Blank et al., 2008; Conneally et al., 1997; Soffer-Tsur et al., 2017; Zonari et al., 2017), though this still remains a translational limitation in the field.

With early successes in differentiating iPSCs into hematopoietic progenitors, further advances in generating downstream immune cell types have reinvigorated the field for clinically applicable, iPSC-based cell therapies. In 2012, T lymphocyte progenitors were generated from iPSC-derived definitive hematopoietic cells, independent of activin/nodal signaling and with potential to drive myeloid, erythroid, and lymphoid differentiations *in vitro* (Kennedy et al., 2012). Shortly after, the generation of iPSC-derived T cells was demonstrated, this time with the addition of engineered CARs targeted to CD19, with anti-tumor activity both *in vitro* and *in vivo*, demonstrating feasibility in using patient-specific T cells for cancer therapies (Themeli et al., 2013). A similar approach was harnessed to generate natural killer (NK) cell-specific-CAR-expressing iPSC-NK cells, with increased anti-tumor efficacy in comparison to primary NK cells and T-antigen-specific CAR-iPSC-NK-cells (Li et al., 2018).

In addition, iPSC-derived NK-CAR-specific NK cells are important for maintaining the populations of cells that may be manipulated for gene editing more easily than their primary cell counterparts, as well as for unlimited generation of NK cells *in vitro* prior to *in vivo* transfusion. By knocking down *CISH*, a negative regulator for IL-15 signaling, in iPSC-NK cells, Zhu et al. demonstrated more efficient metabolic activity and anti-tumor persistence in a xenograft model of acute myeloid leukemia (Zhu et al., 2020). CAR-iPSC-NK cells have numerous alternative benefits, as they are less prone to GVHD than CAR-iPSC-T cells, though both technologies are still being evaluated for safety, efficacy, and scalability for future "off-the-shelf" cell immunotherapies (Depil et al., 2020; Iriguchi et al., 2021). While clinical trials in primary-based NK and CAR-T cell therapies are underway and have already shown success in some cancers, including leukemia (Davila et al., 2014; Park et al., 2018), iPSC-derived alternatives are just beginning to reach phase I/II clinical trials. Fate Therapeutics has been gaining momentum with the development of iPSC-derived NK cells and gene edited iPSC-derived NK cells for treatment of hematological malignancies, among other cancers. These major advancements in cell therapies are expected to reach clinical translation faster than HSC therapies, which can have immense implications in treating not only cancers, but also autoimmune diseases and heart disease, among other applications.

Harnessing new bioengineering tools for hematopoietic regeneration

In the past few years, a number of breakthrough studies have generated great potential for the expansion of HSCs for transplantation. Wilkinson and Yamazaki identified that high concentrations of thrombopoietin, in addition to using polyvinyl alcohol as albumin replacement, resulted in a ~200- to 900-fold expansion of functional HSCs (Wilkinson et al., 2019). This work demonstrates that *in vitro* culture, although heterogenous in its clonal repopulation capacity, may be useful for expanding HSCs prior to transplantation to increase engraftment capability for competitive HSCT. In parallel, Bai et al. showed that using a 3D zwitterionic hydrogel markedly increased the efficacy of HSC expansion *in vitro* by decreasing the production of reactive oxygen species (Bai et al., 2019).

Shah et al. took advantage of ectopic, extramedullary hematopoietic compartments, using engineered bone marrow cryogels to enhance T cell generation in hosts undergoing allogenic HSCT (Shah et al., 2019). At the time of HSCT, cryogels were implanted subcutaneously, serving as a depot for specifying lymphoid progenitors into mature donor CD4+ T cells. These collaborative efforts from the Mooney and Scadden groups demonstrated the use of cryogel scaffolds to enhance donor T cell proliferation and T cell receptor diversification post HSCT, resulting in reduced GVHD and the need for excessive immunosuppressants for HSCT recipients (Shah et al., 2019).

In mechanistic studies of hematopoietic engraftment and differentiation *in vivo*, Dong et al. showed recently the ability to track the differentiation timeline of single HSCs via single-cell transcriptomic analysis, revealing the fate decisions and timelines of transplanted hematopoietic cells, previously inaccessible to HSCT patients (Dong et al., 2020). Single-cell analysis has enabled the discovery of novel transcription factors essential for self-renewal and quiescence in HSCs (Rodriguez-Fraticelli et al., 2020). By using a combination of CRISPR-Cas9 gene editing and transcriptomic analysis, bioengineered systems to study blood and immune cell function can be further optimized to study hematopoietic regeneration in a patient-specific context. These technologies are likely to enable new efforts to expand cord blood- or pluripotent-derived HSCs prior to transplant.

In expanding downstream blood and immune populations for transplantation, maintaining the delicate phenotype of functional iPSC-derived blood and immune cells *ex vivo* remains a

challenge. Recently, Montel-Hagen et al. showed the maintenance and maturation of early T cells derived from iPSCs in artificial thymic organoids, producing naive CD3+, CD8+, and CD3+CD4+ T cells that aligned functionally and molecularly to primary single-positive T cells (Montel-Hagen et al., 2019). In generation of large numbers of iPSC-derived myeloid cells, stirred bioreactors are also being used to generate downstream cells, including macrophages, for eventual use in cell therapies targeting acute infection (Ackermann et al., 2018). Similarly, turbulence-controlled bioreactors were shown to help promote thrombopoiesis and scalable culture of iPSC-derived megakaryocytes and platelets (Ito et al., 2018). Efforts to develop "off-the-shelf" cells at scalable quantities and engineer multi-cellular immune niches to drive maturation *in vitro* are further summarized elsewhere (Smerchansky and Kinney, 2020).

Outlook

Innovative use of biomaterials for regenerative hematopoiesis is starting to allow for *ex vivo* expansion of hematopoietic cells, potentially serving as a test bed for studying disease mechanisms and effects of drugs *in vitro* (Figure 6). For example, the teams of Naveiras and Braschler recently co-cultured MSCs and HSPCs on 3D collagen-coated cryogel particles for efficient cytokine-free hematopoietic culture *in vitro* and subsequent transplantation *in vivo* (Tavakol et al., 2020). With facile aggregation of co-cultured particles and subcutaneous injection without the need for surgery, these engineered niches retained hematopoietic and supported stromal populations for up to 12 weeks in mice. These methodologies suggest new opportunities to study hematopoiesis; however, further validation in human subjects should be conducted to validate the clinical feasibility of these approaches.

OOCs could provide a test-bed for *in vitro studies* of hematopoietic proliferation and engraftment to inform preclinical and clinical trials. Studies of competitive hematopoiesis and whether healthy, regenerative cells can out-perform diseased, malignant cells could be conducted using the OOC models of the marrow (Figure 6). In addition, OOC models provide a powerful tool to understanding the underlying signaling pathways and cellular interactions involved in these processes and shed new light on the mechanisms of repair.

OOC models could help determine the effective timelines for immunotherapy, the need for alignment with the traditional radio/chemotherapy, and the numbers and phenotypical state of antigen-specific immune cells necessary for complete remission of hematological cancers. Though *in vivo* work will continue to be essential, iPSC-derived NK and T cells in tumor spheroid models can provide a toolset for optimizing the route of cell delivery and efficacy, and for studies of the underlying mechanisms of action (Cichocki et al., 2020). In microfluidic OOCs, recent work by Khademhosseini and colleagues demonstrated the efficacious use of an anti-PD-1 immune-checkpoint inhibitor in studying immune-breast tumor spheroid interactions (Jiang et al., 2021). iPSC-derived cells, if used in such integrated immune models, still do not benchmark closely to adult immune responses. Future work must build on these types of studies for design of integrated, multi-organ systems with multiple immune components, like a living bone marrow, for studies of how progenitor and downstream immune-interactions may influence disease models or regenerative strategies in a human context (Graney et al., 2021). Open questions remaining include:

- *By what mechanism, whether by direct cell-to-cell contact or paracrine signaling, do stromal populations interact with hematopoietic cells during health and disease?*
- *What role do extracellular matrix components play in hematopoietic malignancies?*
- *What is the timeline of hematopoietic regeneration, and how do niche components, including endothelial cells and adipocytes, contribute to this regeneration?*
- *How can we mimic both adaptive and innate immune arms in the in vitro models of the blood and immune system?*
- *Can we design testing platforms to study immunotherapies using human biopsies?*

It is advantageous to use engineered hematopoietic models, in tandem with animal models and clinical data, to study the progression of disease, responses to environmental stimuli, and the regenerative capabilities in HSCT preparations in a human-relevant context.

Future perspectives

With major advancements in stem cell biology, bioengineers can now use patient-specific cells to generate human tissues *in vitro*. In combination with natural or synthetic biomaterials, stem cells are being used to engineer both micro- or macro-scale tissues for a variety of purposes. In the future, standardization and scaling up of cell production will be essential for translational advances of larger scale tissues or increased throughput of tissue models. As cells derived from iPSC sources are immature and difficult to maintain *in vitro*, better methods are needed to modulate cell phenotypes into committed, matured cells, and precise benchmarks should be defined (Sharma et al., 2020). Another critical challenge in the use of iPSCs is understanding the effects of their epigenetic and genomic changes during reprogramming, and how these changes might propagate over long periods of time *in vivo* or in OOC models (Pera, 2011).

Engineered systems that can begin to emulate native tissue functions, and in particular OOCs, can be useful in many ways to recapitulate organ phenotypes for drug testing and disease modeling and to study organ- and tissue-level functional characteristics *in vitro* (Figure 1). OOCs with multiple organ systems are of interest for studying organ-organ interactions that are important for studies of systemic conditions. For example, a drug in the body is being metabolized in liver prior to reaching other tissue compartments, a process that could be recapitulated using OOCs. In recent years, there has been an increasing focus on multi-organ interactions, including a number of groups focused on examining the on- and off-target effect of anti-cancer therapeutics in devices with as few as two and as many as ten coupled organs (Chramiec et al., 2020; Edington et al., 2018; Herland et al., 2020; McAleer et al., 2019). The Griffith and Ingber groups have both demonstrated the feasibility of coupling multiple tissue systems by transferring culture medium between tissue compartments, enabling modeling pharmacokinetic-pharmacodynamic relationships. Recently, it was demonstrated that an OOC model of the reproductive tracts was able to produce the hormonal profile of the female menstrual cycle (Xiao et al., 2017). With the increasing interest in sex-specific studies and the recent NIH requirement to include sex as a biological variable, it would be of interest to include hormone

secreting reproductive organs to better emulate female and male physiology.

There is also a need to optimize OOC models to study vascular-tissue interactions, mimic perfusion of blood through a tissue, model drug distribution dynamics, and eventually recapitulate drug and cell infiltration into injury sites. In a model of the blood-brain barrier, Park et al. demonstrated the role of the brain microvascular endothelium, coupled with pericyte and astrocyte supporting cells, in regulating transport of drugs, antibodies, and peptides (Park et al., 2019). George and Kamm have done this effectively for studying tumor metastasis (Jeon et al., 2015; Moya et al., 2013). Since there are significant differences in endothelium phenotype between different tissues, future research would benefit from developing tissue-specific vasculature and mimicking *in vivo* functionality within each tissue compartment (Fleischer et al., 2020).

Using different types of cells and tissues in the same OOC poses a number of challenges, in particular with respect to the use of the same culture medium for all cell/tissue types (common medium). Future work will likely need to find ways to appropriately prepare and selectively maintain essential media compositions to support diverse tissue types.

Although OOC systems offer advantages including humanized and personalized design, animal models will still be essential to understanding the effects of different therapeutic measures on an entire organism and for cognitive and behavioral studies. Parallel studies will be essential in understanding both mechanisms of therapeutic action, by decoupling different variables (OOCs), and complex organism-wide components (animal models); the United States Food and Drug Administration (FDA) is already working toward integrating OOC models within their therapeutic approval pipeline (Ingber, 2020).

As the field grows, interest is developing for integrating immunity into OOCs to increase the biological fidelity, especially in recapitulating disease phenotypes. To date, many microfluidic OOC systems that study immune interactions with a circulatory component have included only one or few cell types, including examples of circulating monocytes (McAleer et al., 2019), neutrophils (Benam et al., 2016), and regulatory/helper T cells (Trapecar et al., 2021). As many studies of physiological regeneration have demonstrated the important roles of the innate and adaptive immune system in regeneration (Chung et al., 2017; Dick et al., 2019; Tang et al., 2017), engineered OOC systems need to recapitulate aspects of immunity in order to effectively test potential adverse reactions to regenerative strategies (Sadtler et al., 2016). Sadtler and Elisseeff have reported on the varying immune responses elicited from both biological and synthetic scaffolds in mice, with responses ranging from a few days to a few months post-implantation showing increasing neutrophil infiltration in synthetic scaffolds, especially those with higher material stiffnesses (Sadtler et al., 2019). Dvir and colleagues have shown the feasibility of generating omentum-based hydrogels with iPSC-derived cells to immunogenically match implants to the donor (Edri et al., 2019). These native biomaterials were assessed for their immunogenicity after incubation with separate fractions of myeloid/lymphoid lineages from the donor. Mimicking these regenerative biomaterial strategies using complex OOC systems with functional immune components would be an effective next step in assessing human responses to regenerative tools.

Despite their utility, OOCs cannot capture complex organism-wide behaviors, unexpected organ-organ interactions, and effects of environmental cues. However, the level of control that can be attained is unparalleled by other methodologies, and thus enables researchers to decouple numerous factors and gain mechanistic insights into tissue homeostasis, disease, and regeneration.

Ongoing considerations:

- *Establishing benchmarks by the scientific community is critical for each OOC system and can help standardize model systems between institutions*
- *"How simple is complex enough"?—choice of model system is dependent on a specific case-by-case application basis and the biological question*
- *Level of maturity for iPSC-derived cells and tissues needs to be defined and reported in models*
- *Choice of iPSC cell sources and population groups can enable personalized medicine; autologous "all iPSC-derived" models may not always be necessary*
- *Advances in stem cell differentiations and biofabrication techniques are required for the fabrication of mature tissues at higher throughput*
- *Development and integration of biosensors and "real-time" readouts is required for serial data collection to obtain a better understanding of regeneration or disease progression*
- *Scaling organ systems in "human-on-a-chip" models will better recapitulate physiological organ size and functional load in the body*
- *Refining which organs or organ systems are necessary to include in "human-on-a-chip" models for studying a specific disease phenotype or drug response—and whether omission of one engineered model may affect the efficacy of a potential therapeutic*
- *Collaborative networks, including funding agencies, can help promote synergy between those developing regenerative therapeutics and those developing models of disease*

Conclusion

Novel and repurposed regenerative therapies are increasingly relying on the information obtained in OOC systems, as these human models offer important validation data prior to early clinical trials. In addition, regenerative therapy can be mechanistically understood using OOCs, as many factors can be decoupled, allowing for systematic understanding of how a treatment is affecting each individual component and what components are the key players in this process. In parallel, OOCs can also be used to test drugs for safety, which in turn would speed up, de-risk, and lower the cost of clinical trials. Patient-specific factors, such as sex and genetic background, can be accounted for in OOC models. These testing platforms can help identify target populations for up-and-coming technologies, as personalized medicine has been emerging in a number of treatment plans. With significant progress in recent years, the use of micro-scale tissue models to study macro-scale organ regeneration is of

increasingly high interest for translation of regenerative technologies.

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DECLARATION OF INTERESTS

G.V.-N is a co-founder of Tara Biosystems, a Columbia University startup company commercializing organs-on-a-chip with human heart muscle, as well as EpiBone, a Columbia University startup company commercializing human bone grafts for regeneration.

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