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Complex Tissue and Disease Modeling using hiPSCs

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http://dx.doi.org/10.1016/j.stem.2016.02.011

Defined genetic models based on human pluripotent stem cells have opened new avenues for understanding disease mechanisms and drug screening. Many of these models assume cell-autonomous mechanisms of disease but it is possible that disease phenotypes or drug responses will only be evident if all cellular and extracellular components of a tissue are present and functionally mature. To derive optimal benefit from such models, complex multicellular structures with vascular components that mimic tissue niches will thus likely be necessary. Here we consider emerging research creating human tissue mimics and provide some recommendations for moving the field forward.

Introduction

It is increasingly clear that animal models fall short in predicting the pathophysiology of many human diseases. Aside from differences in physiology, the immune system, inflammation, and individual genetic backgrounds, there are important differences in liver metabolism compared with other species, even when compared with other primates. These differences impact severity of the disease phenotype and the effectiveness of new drugs in clinical trials. Some of these issues also contribute to the failure to identify potentially toxic side effects of new drugs in current safety pharmacology studies. Drug attrition rates are high not least because present preclinical assays do not always detect potential risk of damage to the heart, kidney, liver, and brain. The emergence of reprogramming as an approach to derive human induced pluripotent stem cells (hiPSCs) from patients and healthy individuals combined with efficient gene modification has led to unprecedented opportunities over the last several years to model human disease. Ten years after their first discovery, many researchers now produce hiPSC lines routinely and induce their differentiation efficiently into multiple somatic cell types that are affected by inherited diseases or are specific drug targets. Commercial providers have optimized reagents and differentiation protocols such that they are now widely applicable across many hiPSC lines. While regenerative medicine is still a long-term goal, other research is looking toward more immediate uses of hiPSCs in understanding mechanisms underlying disease and finding ways to delay or reverse its natural course. Repurposing of existing drugs through better understanding of their mechanisms of action on patient-derived hiPSCs has already resulted in some going directly into clinical trials without intervening animal experiments for severe conditions with no other treatment options. These include amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), and Alzheimer's disease (AD) (Bright et al., 2015; McNeish et al., 2015; Naryshkin et al., 2014; Wainger et al., 2014). This is providing new perspectives for treating intractable conditions at an extraordinary rate.

However, many diseases have multicellular contributions and are not cell autonomous as often assumed. The next generation of disease models is therefore increasingly based on combinations of cell types, sometimes in "organ-on-chip" formats, microfluidic devices that integrate multiple cell types of various developmental lineages as complex synthetic human tissues in chips, or as "organoids," structures of one or more cell types that self-organize into organ subunits. These models can provide rapid readouts of disease pathology and allow the identification of compounds or drugs that could reverse the condition in vivo. They also support integration of various forms of "tissue stress:" inflammatory cytokines and cells, bacterial or viral challenge, biophysical stretch and strain, and microfluidic flow through synthetic vessels, mimicking interaction with the circulatory system.

In this Review we consider what has been learned from various models in which multicellular interaction has been shown to impact phenotype (Figure 1). The emerging complex models that capture the 3D tissue niche and promote cell maturation may represent the future in drug discovery and safety pharmacology models.

Disease Modeling and Safety Pharmacology in Monotypic hiPSC Cultures

hiPSC lines were derived from patients (Park et al., 2008) within 1 year of the first description of human somatic cell reprogramming (Takahashi et al., 2007; Yu et al., 2007). Since then, many hiPSC lines have been described and the cell type expressing the mutant gene has often been shown to exhibit the phenotype expected from the patient in simple monotypic cultures. These have been reviewed both for monogenic (Bellin et al., 2012; Merkle and Eggan, 2013) and complex (Glass et al., 2010; Zhu et al., 2011) genetic diseases so are only described briefly here. The major advantage of studying monotypic hiPSC cultures is that they usually have clear, defined readouts reflecting the pathophysiology of the target cells and the model can be tailored and scaled up as necessary for high-throughput screening. Of note, though, because the first patient hiPSC derivatives were studied before differentiation protocols had become efficient and surface markers available to select differentiated cell types, many studies were carried out in mixed but undefined and variable cell populations, which may have confounded precise phenotypic analysis. However, it is now often possible to create defined cell type combinations that can be controlled from experiment to experiment and are physiologically meaningful. In addition, combinations of cells from different developmental

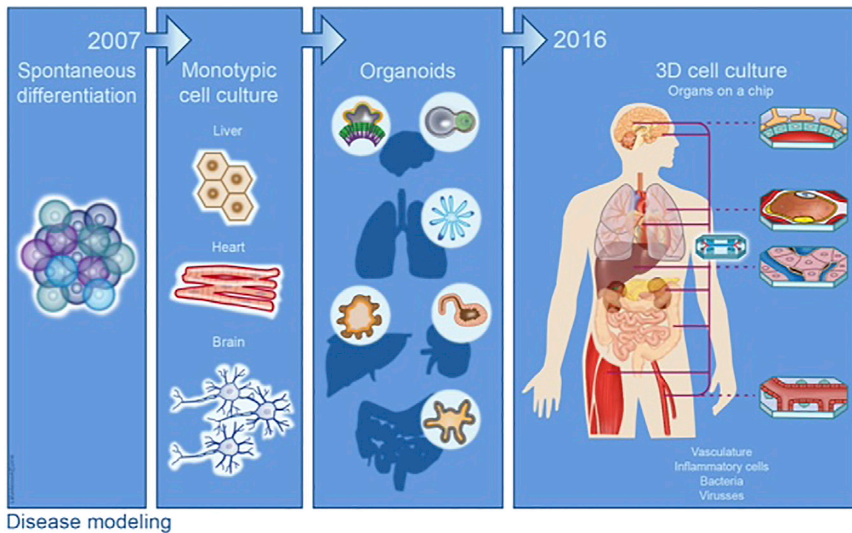

Figure 1. Timeline of Developments in the hiPSC Field
Schematic overview illustrating progress from spontaneous and uncontrolled differentiation of mixed cell types via "embryoid bodies" toward controlled differentiation of specialized cells types in defined conditions that can be used for disease modeling and, finally, the development of complex structures and systems that recapitulate human tissues on a small scale, such as organoids and organ-on-chip technologies for disease modeling applications. The illustration is courtesy of Bas Blankevoort.

lineages can be combined as in normal organs and tissues. We consider here the hiPSC models available for multiple organs but focus on heart and brain since it is now possible to generate different cell subtypes relevant to disease from these organs very specifically, phenotypic readouts are available, and states of cell maturity can be achieved that capture salient features of the diseases in humans.

Heart

Contractile cardiomyocytes represent approximately one-third of the total numbers of myocardial cells but constitute two-thirds of total myocardial volume and are responsible for providing permanent blood flow via coordinated electrical and contractile activity. Disturbances in these properties lead to impaired heart function and are major causes of cardiac disease. Since mutations in genes encoding ion channels (channelopathies) as well as cytoskeletal and sarcomeric proteins (cardiomyopathies) affect cardiomyocyte function in a cell-autonomous manner, these disease phenotypes can be studied both in single cardiomyocytes and in multicellular monotypic cultures.

In the last decade, major advances in standardizing and improving differentiation conditions mean that many hiPSC lines yield cultures containing 20%–80% cardiomyocytes depending on the methodology (Burridge et al., 2012; Mummery et al., 2012). Genetic marking of cardiac transcription factors (Den Hartogh and Passier, 2016) or cardiomyocyte surface markers, such as SIRP1 and VCAM1 (Elliott et al., 2011), enable further enrichment for functional analysis on purified cardiomyocyte populations (Bellin et al., 2013). Conventional single-cell patch-clamp electrophysiology has been widely used to study the effects of ion channel mutations in hiPSC-derived cardiomyocytes (hiPSC-CMs). These ion channels are expressed early during fetal development and in differentiating hiPSC-CMs; phenotypes have therefore been readily detectable despite cardiomyocyte immaturity. Various channelopathies, including long-QT syndrome (LQTS), Brugada syndrome, Timothy syndrome (also called LQT8), and catecholaminergic polymorphic ventricular tachycardia (CPVT) have been modeled in hiPSC-CMs; all faithfully recapitulate cardiac phenotypes observed in patients (reviewed in Karakikes et al., 2015). Cardiomyopathies are also severe heart diseases often caused by mutations in the structural sarcomeric proteins. They are characterized by systolic and/or diastolic dysfunction, sarcomere disarray, hypertrophy (or cardiomyocyte enlargement), and interstitial fibrosis. In single hiPSC-CMs from patients with dilated cardiomyopathy (Sun et al., 2012; Wu et al., 2015), hypertrophic cardiomyopathy (Han et al., 2014; Lan et al., 2013), LEOPARD syndrome, and arrhythmogenic right ventricular cardiomyopathy (Kim et al., 2013; Ma et al., 2013), the disease phenotype manifested as increased cardiomyocyte size in culture, with sarcomeric disorganization and contractile dysfunction.

Drugs can affect cardiomycyte function in much the same way as inherited genetic disorders and induce cardiotoxicity. The majority of cardiotoxic drugs bind to the human Ether-à-go-go (hERG) potassium ion channel, responsible for the repolarizing I_{Kr} currents during the cardiac action potential. The hERG channel block prolongs the action potential (long-QT) and, just as genetic forms of long-QT, can cause life-threatening cardiac arrhythmias. How to predict which individuals might be sensitive to drug-induced long-QT is an important question in drug development. hiPSC-CMs are already proving useful in predicting drug-induced cardiotoxicity but just as importantly, they capture genetic variance and may help identify susceptible individuals for personalized medicine. This was exemplified by the observation that hiPSC-CMs from LQT patients with a mutation in *hERG* showed predisposed sensitivity to inhibitors of the I_{ks} channel (Braam et al., 2013).

Despite these successes of hiPSC-CMs in recapitulating specific cardiac diseases and predicting drug-induced cardiotoxicity, modeling complex multicellular cardiac disease phenotypes requires populations of all cardiac cell types. Besides ventricular, atrial, and pacemaker cardiomyocytes, vascular and epicardial cells are also needed to create complex human heart tissues and mimic their functions. Recent culture condition refinements based on recapitulating the sequence of signals that occur during heart development have enabled all of the major cell types of the heart to be derived from hiPSCs (Birket et al., 2015b; Devalla et al., 2015; Ionta et al., 2015; Iyer et al., 2015; Jung et al., 2014; Witty et al., 2014; Zhang et al., 2011). The stage is now set to start recombining these cells in 2D surfaces pre-patterned to force cardiomyocyte alignment or in 3D spheroids in organoid-like formats to see whether they will undergo maturation and morphogenic organization as in the heart itself.

In 2D, cardiomyocytes plated on polymers, such as polydimethylsiloxane (PDMS) or polyacrylamide, on which rectangles of different aspect ratios (ranging from 1:1 to 7:1) had been patterned, became "anisotropic;" this means they became aligned as in the heart and showed enhanced sarcomere organization (Bray et al., 2008; Ribeiro et al., 2015a). These flexible transparent polymers are relatively soft compared to culture plastic and are more like native heart tissue. They have been used to determine cardiomyocyte contractile force based on displacement of the short edges of the cells or of fluorescent beads on the polymer surface during contraction cycles by video imaging (Ribeiro et al., 2015b). Increased contractile force was recently demonstrated in this way in hiPSC-CMs following the addition of thyroid hormone (T3), insulin-like growth factor (IGF)1, and the corticosteroid dexamethasone. These factors have been implicated during late fetal/perinatal tissue maturation but are produced systemically and not by cardiomyocytes themselves. The increased contraction force was accompanied by increased upstroke velocity of the action potential and reduced resting membrane potential, indicating enhanced maturation under these conditions (Birket et al., 2015a). Of importance, these conditions were crucial for revealing reduced contraction force in hiPSC-CMs derived from patients with cardiomyopathy caused by a mutation in the cardiac protein MYBPC3.

In another approach, hiPSC-CMs can be plated on PDMS muscular thin films (MTFs) micropatterned with fibronectin so that they align. Upon release from the coverslip surface, shortening of cardiomyocytes during contraction causes MTFs to curl, with the displacement reflecting the force of contraction and thus allowing it to be calculated mathematically. MTFs were recently used to demonstrate lower forces of contraction in hiPSC-CMs derived from patients suffering from Barth syndrome, a mitochondrial disorder, that also causes severe cardiomyopathy (Wang et al., 2014).

It is becoming increasingly clear that interactions between multiple cardiac cell types benefit their survival, morphology, maturity, and function. Combined intramyocardial transplantation of hiPSC-derived cardiomyocytes, endothelial cells, and smooth muscle cells, for example, in an IGF-1-containing fibrin patch in a large animal model showed much higher survival of engrafted cells and greater improvement in cardiac function compared to cardiomyocytes alone (Ye et al., 2014). In another study hPSC-derived cardiomyocytes, endothelial cells, and human amniotic mesenchymal cells were combined in a 3D hydrogel. This improved survival and functional performance and changed the molecular profile after 4 and 6 weeks compared to cardiomyocytes alone (Burridge et al., 2014). Multicellular 3D aggregates have also been reported to be better in predicting cardiotoxicity than 2D configurations. 3D aggregates were combined in a microfluidic device and toxic effects of drugs were assessed as alterations in beating frequency by video imaging using an automated detection system (Bergström et al., 2015). Different compounds (doxorubicin, verapamil, and quinidine) were assessed for cardiotoxicity over a period of 6 hr, and although higher sensitivity could be reached using electrophysiology, these assays were efficient and less labor intensive.

Even more complex models are engineered heart tissues (EHTs) in which cardiac cells are combined with biomaterials and non-cardiac cells. EHTs based on collagen/Matrigel scaffolds with neonatal rat cardiomyocytes were first described more than a decade ago (Zimmermann et al., 2000), but have more recently been combined with hPSCs (Soong et al., 2012; Tulloch et al., 2011) or used fibrin/Matrigel scaffolds (Hirt et al., 2014). Although these 3D EHTs subjected to direct mechanical load display higher levels of maturity than standard 2D cardiomyocyte cultures, they are still not equivalent to adult cardiomyocytes. Various approaches reported to promote cardiac maturation in 2D, such as prolonged culture (for several months), the addition of growth factors like IGF and thyroid hormone T3, high oxygen levels, and various combinations of non-cardiomyocytes, also improved maturity of hPSC-CMs in EHTs. Recently, electrical stimulation of hiPSC-CM-derived EHTs for several weeks was shown to increase force generation by 50% and improve structural organization (Hirt et al., 2014). The benefit of 3D culture was demonstrated during the analysis of contractile force in hiPSC-CMs derived from a patient with mutations in the sarcomeric protein titin (Hinson et al., 2015). In 2D culture, no differences were observed between diseased and control cardiomyocytes, but in 3D the difference in contraction force, evidenced as the ability to displace the polymer in which the cardiomyocytes were suspended, was highly significant.

Brain

Neurons are the most important functional components of the brain. Abnormal behavior and function of neurons are considered primary causes of many neurological diseases and psychiatric disorders. Refinement of neural cell differentiation protocols from hPSCs over the last several years now means that pure populations of human forebrain (glutamatergic, presynaptic and postsynaptic cortical, and GABAergic interneurons), midbrain (dopaminergic) and hindbrain neurons, and their pathogenic counterparts are available for the study of mechanisms of (inherited) neural and neuropsychiatric disease initiation and progression (Bellin et al., 2012; Ho et al., 2015). Dysfunction of these neurons is implicated in the pathogenesis of Parkinson's disease (PD), schizophrenia (SZ), autism spectrum disorder (ASD)-like RETT syndrome, epilepsy, and seizure. Aspects of these disease phenotypes are cell autonomous. For example, peripheral neurons from hiPSCs of patients with familial dysautonomia show low expression of IKBKAP, a gene involved in transcriptional elongation. This manifests as defects in neurogenic differentiation and neuronal precursor migration and is corrected by the drug kinetin, which reduces the level of the mutant IKBKAP splice form through modification of mRNA splicing (Lee et al., 2009). In the case of PD, two genetic or familial forms have been described in which the hiPSC-derived dopaminergic neurons show a phenotype: in one, the neurons carried mutations in Leu-rich repeat kinase 2 (LRRK2) and showed increased susceptibility to oxidative stress (Batista et al., 2011), while in the other, there were three copies of the SNCA locus and the neurons showed increased alpha-synuclein protein levels (Devine et al., 2011). While the complex genetic nature of these PD models precludes the generation of isogenic controls by repair of the mutation, rare inherited forms of ALS have been amenable to this and provide a powerful example of the value of monotypic cultures, in this case of motor neurons. hiPSC lines from patients with a rare familial form of ALS caused by mutations in the SOD-1 gene (Wainger et al., 2014; Zhu et al., 2011), which encodes copper-zinc superoxide dismutase and protects cells against

reactive oxygen species, gave rise to derivative motor neurons that showed hyperexcitability compared with their genetically repaired controls. This mimicked the phenotype observed in patients in response to strong magnetic fields. More importantly though, the ALS motor neurons showed an imbalance in Na^+/K^+ by electrophysiology and this could be returned to the levels of that in the isogenic control by an anti-epileptic drug. This drug is already used clinically and is known to pass the blood-brain barrier (BBB). It then took less than 1 year for regulatory approval to repurpose this drug for trial in ALS patients—not just those with the genetic form but also forms with unknown origin because hiPSC motor neurons with an unrelated mutation also showed phenotypic rescue. Thus this approach represents an impressive example of the power of hiPSC disease modeling, particularly in drug repurposing.

Most recent developments include the ability to capture disease phenotypes and drug responses of patients in hiPSC-derived neurons for which the genetic cause is unknown and likely to be complex (Mertens et al., 2015b). However, neuropsychiatric disease is typically associated with deregulation of neuronal connectivity between diverse neuronal populations so that simply studying one neuronal subtype may fail to capture the phenotype of the disease (Spellman and Gordon, 2015). Even in the case of ALS in which the SOD1 gene seems to exert its effect autonomically in motor neurons, there was very early evidence in mice that the effects may be indirect and act by damaging adjacent astrocytes (Bruijn et al., 1997). Similar cell-non-autonomous effects appeared to underlie reduced synaptic puncti in hiPSC-derived motor neurons from patients with SMA (Ebert et al., 2009). SMA is a genetic disease evident in childhood, characterized by motor neuron loss thought to be due to reduced survival motor neuron (SMN). While SMN is expressed ubiquitously, reduced levels in astrocytes may cause their activation and result in the phenotype evident only in hiPSC motor neurons co-cultured with hiPSC astrocytes. The phenotype would again not be revealed without two different neuronal cell types being present. Neural cells from patient hiPSCs can also self-organize into defined neural circuits and 3D systems termed neural organoids. The first striking example of morphogenesis, cell polarity, and neural cell layer formation of neural organoids was seen in the optic cup from mouse ESCs (Eiraku et al., 2011) and later hESCs (Nakano et al., 2012), in which histological sections look remarkably like cross-sections of the eye with the multiple layers of the retina clearly formed. Cerebral organoids from hiPSCs have been used to examine the pathogenesis of neurodevelopmental disorders (Lancaster et al., 2013). These organoids consist of radial glia progenitor cells and neurons (Lancaster and Knoblich, 2014), which recapitulate the gene expression patterns and development of human fetal neocortex (Camp et al., 2015). They are thus potentially valuable models for disorders of neurodevelopment, particularly microencephaly, neurogenesis, and fate specification, conditions not well recapitulated in rodents. In hiPSCs generated from a microencephaly patient with a null mutation in centrosomal protein CDK5RAP2, it was reported that the cerebral organoids were depleted of neural progenitors and showed premature neuronal differentiation, recapitulating features of microencephaly. Finally, telencephalic and cortical organoids containing multiple neuronal cell types have been used to model early development of ASDs. ASD hiPSC-derived organoids were described as showing complex cellular phenotypes that included accelerated cell cycle, upregulation of genes directing gamma-amino butyric acid (GABA) neuron fate, increased synaptogenesis and dendrite outgrowth, and changes in synaptic activity (Mariani et al., 2015).

Future models will most likely incorporate neuronal circuits with, at minimum, two distinct neuronal cell types that form synapses: oligodendrocytes to provide myelination, and astrocytes and microglia to incorporate critical aspects of inflammation and synaptic pruning (reviewed in Haston and Finkbeiner, 2016). These circuits will need repeated stimulation electrically, with stress hormones or relevant drugs, to create clinically meaningful responses. At present hiPSC neurons mimic the molecular and cellular states before symptom onset, so they are presently better suited to study disease predisposition rather than the disease state itself. We refer readers to a recent discussion paper (Brennand et al., 2015) from a group of experts in this area that considers current challenges for creating meaningful patient-specific in vitro models to study brain disorders. The authors concluded that the convergence of findings between laboratories and patient cohorts provides optimism that hiPSC-based platforms will inform future drug discovery efforts, but critical technical challenges remain.

Recent studies demonstrated that reprogramming to the pluripotent state erases the memory of somatic cell origin or "epigenetic memory" but also eliminates the age-related features, such as telomere length and mitochondrial function (Studer et al., 2015). Modeling of age-associated diseases with late onset might be limited using hiPSC technology. Various approaches for "re-aging" hiPSC-derived cells are currently being explored (Cornacchia and Studer, 2015; Studer et al., 2015). The first successful attempts used the expression of progerin that induces a genetic form of premature aging; this effectively "aged" the cells and revealed the phenotype in neurons from PD patient hiPSCs (Miller et al., 2013). A recent study, however, showed that neurons derived by direct lineage reprogramming of somatic cells without an intermediate pluripotent state retained aged features, so age-related cellular defects were revealed without progerin expression (Mertens et al., 2015a).

Genetically engineered hiPSC lines can also be useful in neurotoxicity screening and this can potentially be expanded to other cell types. A powerful cytotoxicity assay has been described using neuronal (MAP2) and astrocytic (GFAP) lineage-specific knockin luciferase reporters (Pei et al., 2015a, 2015b). Interestingly, significant differences in responses were observed in neuronal cells and astrocytes. This further emphasizes the need to have different cell types in cytotoxicity screens for safe-pharmacology applications.

Emerging Principles: Advances from Heterotypic Cultures

As differentiation protocols and the ability to generate monotypic cell populations from hiPSCs improved, it became clear that the earlier mixed cell population that arose spontaneously in aggregates (or embryoid bodies) had several advantages: the differentiating cells created their own microenvironment or niche, which had appropriate organization and produced relevant extracellular matrix proteins. This was lost in the monotypic cultures. Therefore new types of heterotypic cultures began to emerge in which the combinations of cells were better controlled; these

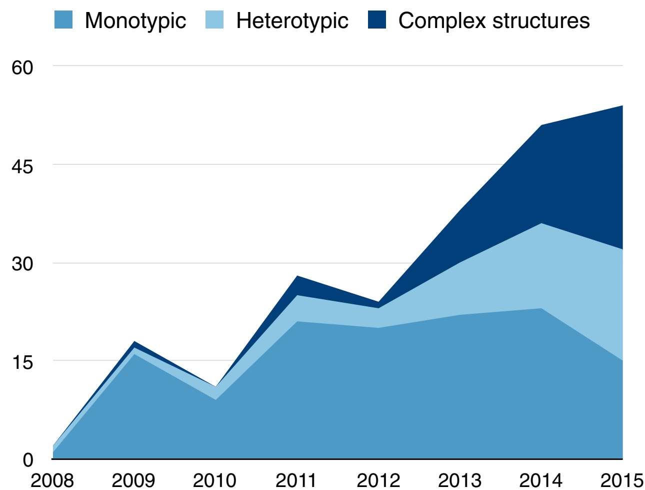

Figure 2. Number of Publications between 2008 and 2016 Describing the Use of Differentiated hiPSC for Disease and "Organ in a Dish" Modeling

PubMed Advanced Search Builder was used for the literature search with the following Builder: [(human pluripotent stem cells) AND differentiation AND (disease modeling)] OR [(human induced pluripotent stem cells) AND differentiation AND disease modeling] OR [(human pluripotent stem cells) AND organoids] OR [(human induced pluripotent stem cells) AND organoids] NOT review. Literature on lineage conversion or human embryonic stem cells was manually excluded. Publications that described the use of hiPSC for modeling neuronal, cardiovascular, and liver disease, as well as 3D organoid formation, were included. Citations were next exported to Papers citation manager and manuscripts were manually assigned a specific color-code for monotypic or heterotypic culture and an additional flag label for complex structures that combine micropatterned, microfluidic, and 2D and 3D microtissues and organoids. The numbers of publications per year for monotypic, heterotypic, and complex structures were then used to create the stacked area plot. The complete list of references included is available in the Supplemental Information. The authors would like to mention a limitation of this graphic representation related to the selection bias.

changes in the field are reflected in the number of publications each year over the last 10 years using embryoid body-, monotypic-, and heterotypic-type cultures (Figure 2).

This is particularly evident in tissues of organs that form later in development than the heart, brain, and vascular system such as the lung, kidney, and liver, which have been challenging to derive from hPSCs. Early successes relied on an exquisite knowledge of embryonic organ development to find the right protocol. The kidney, for example, is derived from intermediate mesoderm in the embryo and ultimately consists of up to 2 million nephrons, the structures that filter the blood, and 20 different cell types that collectively regulate excretion, regulation of pH, and the electrolyte and fluid balance. The liver is an example of how heterotypic cultures may lead to better liver tissue models based on hiPSCs. The liver is the largest internal organ in the human body. It regulates over 500 different functions that include metabolism of fats and proteins, bile production, excretion of drugs and hormones, and blood detoxification (Bhatia et al., 2014). Liver has a very complex architecture with hepatocytes organized as cords with other cells, including sinusoidal endothelial cells and biliary epithelial cells or cholangiocytes, positioned between the cords and organized as bile ducts.

Here we will discuss recent advances in generation of complex kidney and liver structures from hiPSCs.

Kidney

Drug nephrotoxicity is an important cause of acute kidney injury in hospitalized patients and currently there are no patient-specific models for assaying nephrotoxicity in vitro. An important advance in this context is the recent generation of complex kidney-like structures from hiPSCs (Morizane et al., 2015; Takasato et al., 2015). By controlling the timing of patterning within the hiPSC-derived intermediate mesoderm, it was recently shown that complex kidney organoids containing nephrons associated with a collecting duct network and surrounded by renal interstitium and endothelial cells could be generated. Comparison of transcriptional profiles of kidney organoids with human fetal tissues showed that they were very similar to first trimester human kidney (Roost et al., 2015). Since patient-derived hiPSCs could provide such opportunities, there has been significant interest in the observation that despite their immaturity, proximal tubules from hiPSCs showed the ability to endocytose dextran and differentially apoptose in response to cisplatin, an anticancer drug with proximal and distal tubular toxicity (Takasato et al., 2015), as well as to gentamicin, a commonly used antibiotic with proximal tubular toxicity (Morizane et al., 2015). In addition, several genetic kidney diseases have been introduced into hiPSCs using CRISPR/Cas9 to target relevant genes, creating isogenic pairs for direct comparison (Freedman et al., 2015). Cyst formation by kidney tubules in the hiPSC organoid model was disrupted when the polycystic kidney disease genes PKD1 or PKD2 were deleted. This was clearly distinct from effects on epithelium surrounding the lumen in epiblast spheroids from the same hiPSC lines; here the capacity to form lumen at these earlier stages of hiPSC differentiation was unaltered by the mutations. In addition, a clinical biomarker of proximal tubule injury, kidney injury molecule-1 (KIM-1), was upregulated only in the kidney organoids and not in hiPSC epiblast, indicating that the response was tissue specific and likely required the complex, multicellular 3D context. Although these structures exhibit lineage complexity that differs from conventional kidney cell lines and organoids, all cellular components of the developing proximal nephron (tubular cells, endothelial cells, nephron progenitors, and podocyte-like cells) are present in a well-organized way within each individual organoid.

Liver

During development, hepatic endoderm cells delaminate and form liver bud. Endothelial cells are essential for the initiation of the liver bud formation from hepatic endoderm, proliferation of the hepatic cells, and hepatic maturation (Matsumoto et al., 2001). In addition, inductive signals from endothelial cells also promote liver regeneration and hepatocyte proliferation upon injury (Ding et al., 2010, 2014; Hu et al., 2014). Furthermore, the presence of the vascular structures and blood flow is essential for the maturation of the hepatocytes in zebrafish (Korzh et al., 2008). Recently, Takebe et al. reported generation of functional human liver by co-culture of hiPSC-derived hepatic endoderm cells with the stromal cells composed of primary human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (hMSCs) (Takebe et al., 2013). The self-organized liver buds exhibited increase expression of mature hepatic markers as early as 6 days in co-culture. Furthermore, transplantation of these heterotypic structures into mouse resulted in rapid anastomosis with the host vasculature and formation of functional liver and extended expansion of

hiPSC-hepatocytes over a period of 2 months. More importantly, these hiPSC-derived mini-livers contained hepatocytes organized into the hepatic cord-like structures with characteristics of adult liver. Simultaneous induction of liver organoids upon pulse induction of GATA6 over a period of 5 days was reported recently (Guye et al., 2016). The GATA6 inductive approach resulted in the formation of definitive endodermal cells with hepatic characteristics, as well as mesoderm and neuroectoderm-derived vascular, hematopoietic, and neuronal cells. Prolonged culture over a period of 30 days resulted in the formation of functional tissues with a layer of hepatocytes and vascular cord-like structures. Therefore, this approach can be used for simultaneous induction of different cellular components from hiPSCs, which would be beneficial for transplantation studies and development of novel disease models.

Heterotypic interaction of NOTCH2 expressed on cholangiocytes with mesenchymal JAG1 is needed for the development of the bile duct structure (Hofmann et al., 2010). Recapitulation of these developmental principles facilitated successful differentiation of cholangiocytes from hiPSC-derived hapatoblasts via co-culture with JAG1-expressing stromal cells, OP9 (Ogawa et al., 2015). Hollow cyst-like structures were then generated by co-culture of aggregated hiPSC-derived hepatoblasts with OP9 cells in a 3D matrix of collagen and Matrigel. In addition, 3D conditions facilitated efficient tubulogenesis and growth of the aggregates. Interestingly, the cyst-like tubular structures were also generated without stromal cell layer if (adult stem cell) organoid-promoting culture conditions in 3D were used (Sampaziotis et al., 2015). In both cases, formation of the cyst-like structure was abrogated by NOTCH inhibition with γ-secretase inhibitor, indicating its importance in the maturation of the bile duct from hiPSC-derived cholangiocytes. Strikingly, both approaches resulted in functional cholangicytes with disease phenotypes when they were generated from patient hiPSCs with inherited polycystic liver disease or cystic fibrosis (CFTR F508del mutation). Administration of the drug (VX908), which is already in clinical trials for cystic fibrosis, resulted in the correction of the disease phenotype, demonstrating that this technology can be used to screen for potential therapeutic agents for bile disorders.

Other chronic liver diseases for which efforts are ongoing to create hiPSC-based models include cirrhosis, caused by alcohol abuse, drugs, virus infection, inflammation, or autoimmune and metabolic conditions, which accounts for more than 1 million deaths worldwide annually; and multiple inherited conditions that can cause liver damage such as alpha-1-antitrypsin deficiency, hemochromatosis, Wilson's disease, hereditary tyrosinemia, cystic fibrosis, and polycystic liver disease. A drawback at present to using hiPSC-derived liver cells in drug target discovery is that liver cell types other than hepatocytes, like oval cells, that would ideally be required for optimal disease and tissue modeling are not yet available. Progress here guided by developmental biology principles has a high likelihood in driving interest in adopting hiPSCs for this purpose.

Other Organs

Organoids are multicellular 3D structures or organs in miniature. They were until recently associated with adult stem cell cultures from tissues of the gastrointestinal tract (Huch and Koo, 2015; Johnson and Hockemeyer, 2015; Sato and Clevers, 2013). These organoids have now been derived from large and small intestine, pancreas, liver, stomach, and prostate. They generally contain the epithelial component of the tissue from which they are isolated in highly organized structures, but not the stromal tissue or vasculature. Organoids derived more recently from hiPSCs, however, often contain multiple tissue cell types as well as stromal cells and vasculature and are therefore considered heterotypic. Examples include (ectodermal) hiPSC-neural organoids, as discussed earlier, (mesodermal) hiPSC-kidney organoids, and (endodermal) hiPSC-intestinal, lung, gastric, and liver organoids (Dye et al., 2015; Lancaster et al., 2013; McCracken et al., 2014; Paşca et al., 2015; Spence et al., 2011; Takasato et al., 2015). If given the proper extracellular matrix, these structures show a remarkable ability to self-organize and develop the polarity seen in normal tissue. Organoids based on hiPSCs offer particular opportunities for disease modeling only partially met by adult-stem-cell-derived organoids: adult stem cells give rise to organoids with more mature phenotypes, but in general only the epithelial component of a tissue is represented.

What Are the Next Steps? Increasing Complexity and Multiple Integrated Readouts

Inclusion of Vasculature

Blood vessels not only supply tissues with nutrients and oxygen; they are also intimately involved in regulation of tissue morphogenesis, regeneration, and homeostasis, including the resolution of inflammation. During early development endothelial cells also play an important role in organogenesis. They provide instructive signals during heart morphogenesis and septation, are essential for the maturation of cardiomyocytes, and instruct development of endoderm-derived organs, such as liver, lung, kidney, and pancreas (Cleaver and Melton, 2003; Ding et al., 2014; Kao et al., 2015; Ramasamy et al., 2015).

Inclusion of the vasculature would therefore be an important next step in recreating complex tissue structures from hiPSCs. This was recently illustrated by a study in which "organ buds" made up of hPSC-derived tissue-specific progenitor cells were combined with endothelial cells and mesenchymal stromal cells (MSCs). The MSCs initiated condensation within the heterotypic cell mixtures. By defining optimal mechanical properties of the matrix, transplantable 3D organ buds could be formed from tissues that included kidney, pancreas, intestine, heart, lung, and brain. These organs buds were vascularized in vivo and self-organized into functional, tissue-specific structures (Takebe et al., 2015). Of note, each tissue and organ has its own type of endothelial cell, so tissue-specific blood vessel induction in these structures may be of importance (reviewed in Rafii et al., 2016; Ramasamy et al., 2015).

Aside from performing instructive roles in tissue morphogenesis, endothelial cells form vascular tubes, which require "mural cells" (pericytes and vSMCs) to develop into stable vasculature. Defective interactions between these cells underlie different genetic disorders that can cause hemorrhages and also be the cause of a spectrum of neurological conditions, such as hereditary hemorrhagic telangiectasia (HHT), cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and retinal vasculopathy with cerebral leukodystrophy (RVCL) (Yamamoto et al., 2011). Blood vessels "in a dish" that recapitulate endothelial-mural cell interactions would benefit investigation of mechanisms underlying these disorders, and they could also be used to study other

pathologies, such as thrombosis and vascular malformation disorders like cerebral cavernous malformations (CCMs) and others (Gibson et al., 2015; Storkebaum et al., 2011; Westein et al., 2013). Both 2D and 3D systems with multiple vascular cell types present would be essential in modeling these diseases.

Modeling Inflammation

Inflammation is a self-defense mechanism that protects organisms from infection and tissue injury. Chronic inflammation resulting from persistent infection and prolonged activation of the endothelium causes many pathological conditions, including cardiovascular, neurological, and neurodegenerative diseases. These are of particular concern because of their poor prognosis, significant morbidity, and the lack of effective treatments. In developing hiPSC disease models, especially for chronic and inflammatory conditions, it is therefore essential to take endothelial and inflammatory cells into account.

Modeling inflammatory responses is complex, and it requires assessment of interaction between different cells in the tissues, such as inflammatory cells, resident macrophages and microglia in the brain, endothelial cells, and epithelial cells in the lung and intestine. Through recent advances in hiPSC technology it has now become possible to differentiate many of the cellular components that would be useful to model inflammation in vitro. These include neutrophils, monocytes/macrophages, and microglia (Lachmann et al., 2015; Nayak et al., 2015; Schwartz et al., 2015). These cells serve as an initial wave of infiltrating cells at the site of tissue damage or infection and can exacerbate the inflammatory response. Interestingly, a comparison of hiPSC-derived macrophages with primary isogenic cells derived from peripheral blood demonstrated high phenotypic, functional, and transcriptomic similarity (Zhang et al., 2015).

Several groups have shown that endothelial cells from hiPSCs exhibit functional inflammatory responses (Adams et al., 2013; Belair et al., 2015; Patsch et al., 2015). hiPSC-derived endothelial cells also exhibit robust responses to proinflammatory stimuli (TNFα, IL-1β, and LPS) reflected in increased surface expression of adhesion receptors E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1). hiPSC-endothelial cells can bind human leukocytes under static conditions (Patsch et al., 2015) and under physiological flow that mimics blood in vivo (Adams et al., 2013). Derivation of autologous endothelial cells and leukocytes would be a major step forward in modeling uncontrolled inflammatory reactions with patient-specific cells. In particular, these might be useful for the pharmaceutical industry as assays for adverse drug responses (ADRs), much like those based on autologous primary human cells (Reed et al., 2015). The extreme ADR in the TGN1412 (an immunomodulatory drug) clinical trial that caused multiple organ failure in six young, healthy participants could perhaps have been avoided if a representative human in vitro assay had been available.

hiPSC-derived lung or intestine cells would be useful in modeling severe influenza reactions, Crohn's disease, and inflammatory bowel disease (IBD). A recent paper based on mouse intestinal stem cells would argue similarly for an important role of the immune system in promoting intestinal regeneration (Lindemans et al., 2015).The functionality of hiPSC-derived lung epithelial cells has recently been demonstrated in the case of life-threatening influenza reactions that occur in otherwise healthy children. The hypothesis that genetic factors could underlie influenza sensitivity was proposed some time ago and reviewed recently (Casanova, 2015). hiPSCs derived from a child with the severe influenza were used to demonstrate that the defective interferon (INF) response in lung-epithelial cells caused by a mutation in INF regulatory factor 7 (IFN7) is responsible for increased virus replication (Ciancanelli et al., 2015). Protocols to differentiate hiPSCs into appropriate lung cells made it possible to confirm that the severity of influenza might be due to inborn genetic errors in immunity, and that it might also be possible to develop therapies based on hiPSC models. Other conditions are similarly awaiting new developments in virus or bacterial hiPSC interaction.

Modeling the Blood-Brain Barrier

It is thought that a contributory factor to neurovascular disease may be initial vascular dysfunction that leads to the breakdown of the BBB. Vascular contributions to the development of cognitive impairment and dementia have become recognized over the last several years (Gorelick et al., 2011; Storkebaum et al., 2011; Zhao et al., 2015) and changes in the vasculature often precede neuronal defects. Patients with brain blood vessel malformations are at risk of developing neurological symptoms, usually because of hemorrhages, the incidence of which increases with age (Söderman et al., 2003). A vascular contribution is even becoming evident in AD. Deposition of β-amyloid (Aβ) peptide on the walls of brain capillary vessels, for example, is the common pathology of a condition called Cerebral Amyloid Angiopathy (CAA) (Hendriks et al., 1992), a major cause of hemorrhagic stroke in the elderly not associated with hypertension. Multiple recent studies also showed that CAA could cause further cognitive impairment, AD, or dementia. Other vascular pathologies have also been indicated to precede AD (Kanekiyo et al., 2014; Verghese et al., 2011). Understanding the mechanisms of neurovascular dysfunction and mechanisms that regulate BBB is therefore important for prevention and identification of potential drug targets for neurovascular diseases.

There are three major cellular components of the BBB: endothelial cells, pericytes/vSMCs, and astrocytes, which together form the specialized barrier that isolates and protects brain parenchyma from harmful components of the blood, facilitates active transport of nutrients, and mediates clearance of waste. Astrocytes and pericytes provide critical signals for the maturation and maintenance of the BBB and are thought to facilitate induction of BBB-like characteristics in endothelial cells. Therefore incorporation of all these components will be essential to recreate the BBB in vitro. Recently Lippmann et al. demonstrated that co-culture of rat neonatal astrocytes or human neuronal progenitor cells (NPCs) can be used to direct hiPSC endothelial cells to differentiate into BBB-like endothelial cells (Lippmann et al., 2012, 2014). Derivation of mature astrocytes from hiPSCs has been reported (Roybon et al., 2013; Sareen et al., 2014). Systems in which all of the cellular BBB components were derived from hiPSCs would be extremely valuable in identifying the causative cellular components in the disease pathology. Furthermore, inflammatory components could be incorporated via either reactive astrocytes stimulated with pro-inflammatory factors (TNFα, IL-1β, and IFNγ) (Roybon et al., 2013) or hiPSC-derived microglial or "brain resident macrophages" that could again be derived from hiPSCs (Schwartz et al., 2015).

Blood flow simulation and peripheral immune cells can be also incorporated as challenges by integration in micron-scale hollow tubes lined with endothelial cells separated from the pericyte/astrocyte or microglial components by thin porous membranes. Several prototypes of these ''BBB-on-a-chip'' devices have been described over the last 2 or 3 years (Booth and Kim, 2012; Brown et al., 2015; Cho et al., 2015; Deosarkar et al., 2015; Griep et al., 2013; Hyun Jo et al., 2015; Yeon et al., 2012). Future prototypes would incorporate not only the BBB compartment, but also a cerebral spinal fluid (CSF) compartment, currently being developed as part of the NIH-funded brain-on-chip program (Alcendor et al., 2013).

Additionally, sensors to measure transendothelial barrier resistance (TEER), electrical sensors to quantify infiltrating leukocytes, and label-free microbiosensors to measure amyloid beta isoforms, protein transport, or drug delivery across BBB are developing. These will transform the field and reduce the use of animal models in assessing the ability of drugs to cross the BBB.

Organs-on-Chips

Organs-on-chips are an emerging technology with excellent potentials for increasing tissue complexity of hiPSC tissue models and including vasculature. There are major initiatives in the US to promote the technology (https://ncats.nih.gov/; http://wyss.harvard.edu) as well as national initiatives in Switzerland (http://www.artorg.unibe.ch) and the Netherlands (http://www.hdmt.technology/). Organs-on-chips are microfluidic devices (or ''chips'') about the size of a microscope slide usually made of a transparent polymer and containing one or more small open or closed culture chambers (or micro-incubators) coupled to small (microfluidic) channels through which (culture) fluid or gas can flow. The cells, once seeded, proliferate or differentiate as in normal cell culture but may also mature or age because of a more physiological microenvironment than regular culture conditions. Organs-on-chips mimic the smallest functional subunits of human organ or tissue: the alveolus of a lung (lung-on-chip), synchronously contracting heart cells (heart-on-chip), intestine (gut-on-chip), and the like in a (micro)environment similar to that in vivo (Bhatia and Ingber, 2014; Wilmer et al., 2016). There is particular potential here for hiPSCs since these are now amenable to stable integration of reporter constructs (Den Hartogh and Passier, 2016). Most of the early organs-on-chips were based on primary cell cultures or transformed cell lines (Alonzo et al., 2015; Bertassoni et al., 2014; Bischel et al., 2013; Jeon et al., 2014; Kim et al., 2016; Moya et al., 2013; Nguyen et al., 2013; Sackmann et al., 2014; Wang et al., 2016; Zervantonakis et al., 2011, 2012; Zheng et al., 2012). Incorporation of hiPSC derivatives from patients or healthy individuals (or a combination of the two) is now widely considered. In particular, microfluidic models are now starting to be used to create vascular models with hiPSC derivatives (Belair et al., 2015; Mathur et al., 2015; Palpant et al., 2015; Theodoris et al., 2015; Wanjare et al., 2015). We recently used these microfluidic chambers to create 3D blood vessels from hPSC-derived endothelial cells and pericytes (van der Meer et al., 2013). Inclusion of cardiomyocytes into organ-on-chip devices that mimic blood flow and the endothelial-cardiomyocyte interface can improve prediction of drug-induced cardiotoxicity (Mathur et al., 2015). Incorporation of endothelial cells and the creation of heterotypic microphysiological systems are already dictating new directions in the field (Kurokawa and George, 2016). The open microfluidic systems also allow collection of the ''flow through'' for analysis of secreted proteins.

Finally, attempts are ongoing to differentiate hiPSCs directly in microfluidic devices. This depends on spatiotemporal control of the microenvironment by optimal delivery of exogenous factors and removal of cell-secreted factors under controlled perfusion frequency (Giobbe et al., 2015). This technology is again dependent on the intrinsic self-organization of hiPSCs to generate organotypic cultures and could mean that fewer cells would be needed to form functional microscale structures. Functional cardiac and hepatic cells have thus been obtained within 2 weeks of seeding and these showed expected drug responses in situ.

Organs-on-chips can thus yield unique biomedical data from hiPSCs through the integration of multicellular and multifactorial aspects of tissue physiology and disease.

Integrating Molecular and Functional Readouts

In all of these models, the greatest benefit will accrue if they support long-term, real-time analysis, including gene-based (fluorescent) reporters and electrical, mechanical, and bio-nanosensors for toxicity and disease (as both end-point and mechanistic readouts). Including genome-wide molecular analysis (genomics, transcriptomics, proteomics, and metabolomics) will be of importance but access will be required for appropriate (preferably repeated) cell sampling. Alternatively, as the sensitivity of detection methods for proteins increases, microfluidic devices in particular lend themselves to continuous monitoring of proteins in flow-through medium, and proteins secreted by cells developing disease phenotypes, challenged with drugs, or undergoing the effects of stress can be determined. Such (patho-)physiological information can normally only be obtained through animal testing. Systems biology approaches based on computational modeling will be required to integrate data from different hiPSC derivatives, establish relationships between the data, and identify clinically relevant endpoints. The importance of the multidisciplinary methodologies and expertise for this is beginning to be widely recognized.

Future Outlook

A key outstanding question for the field is whether hiPSCs will ultimately prove to be useful for disease modeling and drug discovery given what we now know about the challenges they pose. In addition and in light of rapidly evolving CRISPR technologies, human ESCs may turn out as the easier-to-standardize model system for studies of monogenetic diseases even though they have the disadvantage that information on the severity of the phenotype, age of onset, and drug responsiveness would not be known. Nevertheless, hiPSCs will likely be of special use as patient-specific reference models and/or be exploited in modeling complex diseases or diseases for which causative mutations are unknown.

Issues that still need to be addressed include the lack of mature phenotypes in both hESC and hiPSC derivatives, the extent of improvement over animal models for drug discovery, and the degree of complexity that can be recapitulated with in vitro stem cell models. To derive optimal benefit, it will be important for the field to focus on developing deeper complexity in these models and define useful readouts to enable their utility in drug discovery and safety pharmacology. An indirect outcome could be reduction in the use of laboratory animals because the alternatives represent better human mimics.

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Questions and issues that need to be addressed in the future include:

- Do we need to integrate multiple organs on one chip? What are the advantages and the disadvantages?
- Will complex, highly advanced human models lead to a better predictability (compared to current models and human hPSC-derived high-throughput models)?
- Where will models fit in the process of drug discovery? It may be important to implement models at different stages during this process.
- How will possible findings from human hPSC-derived models be best linked with clinically relevant data?
- In the context of reproducibility and standardization, it is important to follow "Good Cell Culture Practice".
- Communication between the different stakeholders (scientists, industry, international regulatory bodies, and socially engaged organizations) is required for successful implementation of hPSC-derived models in the process of drug discovery and safety pharmacology and for the replacement of animal models.
- The relevance for regenerative medicine will need to be considered (for example, optogenetics in combination with hPSC-derived grafts in a Parkinson's disease model) (Steinbeck et al., 2015).

Multitypic cell cultures from hiPSCs could ultimately lead to better and safer drugs and a better understanding of human disease.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes a detailed list of the references used to compile Figure 2 and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2016.02.011.

CONFLICTS OF INTEREST

Christine Mummery and Robert Passier are co-founders of Pluriomics bv. Christine Mummery is on the advisory board of Galapagos bv.

ACKNOWLEDGMENTS

This Review discusses a rapidly growing area of research and the authors apologize to those many other authors whose papers may not have been cited here. Research from the authors' laboratories is supported by the European Research Council (ERCAdG 323182 STEMCARDIOVASC), European Community's Seventh Framework Programme (FP7/2007-2013) grant agreement 602423, and ZonMw-MKMD-40-42600-98-036.

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