High-Throughput Screening Enhances Kidney Organoid Differentiation from Human Pluripotent Stem Cells and Enables Automated Multidimensional Phenotyping

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In Brief
Organoids derived from human iPSCs have great potential for drug screening, but their complexity poses a challenge for miniaturization and automation. Freedman and colleagues establish a robotic pipeline to manufacture and analyze kidney organoids in microwell arrays. They apply this system to improve differentiation, measure toxicity, and comprehend disease.

Highlights
- Liquid-handling robots generate and analyze kidney organoids in microwell arrays
- Single-cell RNA-seq reveals that organoid cell types recapitulate human kidney complexity
- Growth factor addition greatly increases vascular endothelial cells in organoids
- A phenotypic drug screen discovers a role for myosin in polycystic kidney disease
High-Throughput Screening Enhances Kidney Organoid Differentiation from Human Pluripotent Stem Cells and Enables Automated Multidimensional Phenotyping

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INTRODUCTION

Organoids derived from human pluripotent stem cells are a potentially powerful tool for high-throughput screening (HTS), but the complexity of organoid cultures poses a significant challenge for miniaturization and automation. Here, we present a fully automated, HTS-compatible platform for enhanced differentiation and phenotyping of human kidney organoids. The entire 21-day protocol, from plating to differentiation to analysis, can be performed automatically by liquid-handling robots, or alternatively by manual pipetting. High-content imaging analysis reveals both dose-dependent and threshold effects during organoid differentiation. Immunofluorescence and single-cell RNA sequencing identify previously undetected parietal, interstitial, and partially differentiated compartments within organoids and define conditions that greatly expand the vascular endothelium. Chemical modulation of toxicity and disease phenotypes can be quantified for safety and efficacy prediction. Screening in gene-edited organoids in this system reveals an unexpected role for myosin in polycystic kidney disease. Organoids in HTS formats thus establish an attractive platform for multidimensional phenotypic screening.

SUMMARY

Organoids derived from human pluripotent stem cells are collections of cells in vitro that resemble a bodily organ in structure and function. These next-generation cell-architecture systems remain highly accessible to experimental manipulation and analysis but are also sufficiently complex to model tissue-scale development, injury, and disease (Freedman et al., 2015; McCracken et al., 2014). Human organoids have now been derived representing intestine, kidney, eye, and other organs (Freedman et al., 2015; Hayashi et al., 2016; McCracken et al., 2014; Morizane et al., 2015; Spence et al., 2011; Taguchi et al., 2014; Takasato et al., 2015). Many types of organoids can only be derived from human pluripotent stem cells (hPSCs), the cultured equivalents of the early embryonic epiblast, from which all somatic tissues differentiate (Thomson et al., 1998). As hPSC-derived organoids can be generated from any patient, they have great potential for immunocompatible tissue replacement therapies and prediction of individualized outcomes in human clinical populations (Dekkers et al., 2013; Huang et al., 2015; Takahashi et al., 2007).

An attractive potential application is to utilize organoids for automated, high-throughput screening (HTS) of hundreds of thousands of chemical compounds or genes simultaneously, at a scale that could not be accomplished in mammalian model organisms (Major et al., 2008). In contrast to the simple cell cultures typically used for HTS, organoids are capable of reconstituting features of complex disease, such as PKD and brain microcephaly (Cruz et al., 2017; Freedman et al., 2015; Lancaster et al., 2013). Organoids derived from highly regenerative somatic stem cells, such as intestinal crypt cells or mammary cancers, have previously been generated in HTS-compatible formats, to enhance these cultures and identify modifiers of disease (Gracz et al., 2015; Sachs et al., 2018). However, organoids representing many organs can only be derived from hPSCs, involving three-dimensional growth conditions, lengthy stepwise differentiation steps, and special processing for immunofluorescence, all of which pose significant challenges to automation and miniaturization (Freedman et al., 2015; Hayashi et al., 2016;
McCracken et al., 2014; Morizane et al., 2015; Spence et al., 2011; Taguchi et al., 2014; Takasato et al., 2015). For this reason, HTS involving hPSC derivatives has been limited to simpler cultures, such as cell monolayers, which are restricted in their capacity to model complex tissue phenotypes (Chen et al., 2009; Doulatov et al., 2017; Pagliuca et al., 2014; Sharma et al., 2017; Yang et al., 2013). Here, we describe protocols for the miniaturization and automation of human organoid differentiation from hPSCs, using the kidney as a representative organ lineage. We further demonstrate the feasibility of using this system to enhance organoid differentiation and model disease.

**RESULTS**

**Differentiation of hPSCs into Organoids in HTS Formats**

To generate organoids compatible with HTS, hPSCs were plated in 96- and 384-well formats and differentiated into the kidney lineage for 3 weeks (Figure 1A). Kidney organoids are highly complex and of great biomedical interest for their potential to model disease, toxicity, and regeneration (Freedman et al., 2015; Morizane et al., 2015; Taguchi et al., 2014; Takasato et al., 2015). To establish protocols accessible to a broad range of laboratories, we prepared plates either manually, using multi-channel pipettes, or automatically, using liquid-handling robots to perform all steps of plating, differentiation, fixation, and phenotyping (Figure 1A).

Following differentiation, each well contained numerous kidney organoids, as detected by *Lotus tetragonolobus* lectin (LTL) binding of the proximal tubular segments (Figure 1B). In addition to proximal tubular cells, each organoid included distal tubule (ECAD+) and podocyte (NPHS1+) cell populations in distal-to-proximal arrangements (Figure 1C and Video 1).

To simplify automation, we adapted for HTS a differentiation protocol that involves only a single induction step with the kinase inhibitor CHIR99021 (Freedman et al., 2015). In side-by-side experiments, the nephron-like structures within the organoids derived using this protocol closely resembled those observed in organoids derived using protocols from other groups (Figures S1A and S1B) (Takasato et al., 2015). To accommodate high-throughput applications, differentiation was performed on standard tissue culture plates as previously described in larger wells (Freedman et al., 2015), rather than transwell plates or suspension cultures. This resulted in the formation of numerous kidney organoids per well, each organoid containing ~5 nephron-like structures and growing to a natural size of ~200 μm in diameter (Figure 1C and Video 1), similar to intestinal organoids (Dekkers et al., 2013; Spence et al., 2011).

As these cultures were spread out in two dimensions, the kidney organoids formed discrete nests of tubules that could be clearly discerned from surrounding, non-kidney cells with standard microscopes. This contrasts with other differentiation protocols in which the entire culture, including both kidney and non-kidney cells, is dissociated and re-aggregated into a three-dimensional pellet of arbitrary size, also called a kidney organoid (Morizane et al., 2015; Taguchi et al., 2014; Takasato et al., 2015). We found that such dissociation and replating steps were unnecessary for kidney organoid generation and instead resulted in dramatically fewer and smaller tubular structures (Figures S1C and S1D). In addition, we previously established genetic models of PKD and nephrotic syndrome in our adherent organoid cultures, making these organoids the natural starting point for HTS experiments focusing on disease (Cruz et al., 2017; Freedman et al., 2015; Kim et al., 2017).

**Optimization of Organoid Differentiation with Microwell Plates**

We used HTS organoid plates to quantitatively assess differentiation conditions and optimize their own production. As
CHIR99021 concentrations and cell numbers, with 3,000 cells/well and 14 μM CHIR99021 producing the optimal number of organoids per well (Figure S2B).

We further utilized our organoid HTS platform to quantify the effect of CHIR99021 on organoid differentiation and substructure over a broader range of concentrations, ranging from 0 to 20 μM. For these experiments, the software for our IN Cell Analyzer (GE Healthcare) was adapted to recognize organoids as structures containing proximal tubules, distal tubules, and podocytes in close proximity, enabling analysis of these individual subcompartments within each organoid (Figures 2A and S2C). Differentiation was quantified in three different subclones of WA09 hPSCs (Figure 2B). Each differentiated well of a 384-well plate typically contained ~10 organoids, representing ~50 nephron-like structures (Figures 2A, 2B, S2C, and S2D). These experiments revealed an ideal range of CHIR99021 concentrations capable of generating organoids (Figure 2B).

Surprisingly, the optimal CHIR99021 concentration varied significantly for each individual subclone, despite the fact that these were all derived from the same original hPSC line (Figure 2B). Z' factor calculation indicated that line 1 was excellent (Figure 2B). The optimal number of cells for the initial plating varied from ~1,000 to ~3,000, depending on the experiment, likely owing to stochastic differences between the cells from passage to passage (Figure S2D). When implanted into immunodeficient animals, each of these subclones formed large teratoma growths containing tissues from the three embryonic germ layers, demonstrating that they remained fully pluripotent (Figure S2E).

By performing quantitative image analysis with the IN Cell Analyzer, we further examined the proportion of each organoid that contained proximal tubules, distal tubules, or podocytes. These experiments revealed a tight correlation between the proportions of these three nephron segments throughout the active range of CHIR99021 concentrations (Figure 2C). A slight increase in the number of podocytes was observed in middle and high CHIR99021 concentrations, relative to the lowest doses (Figure 2C). Even at concentrations of CHIR99021 that produced very few organoids, the organoids that did form had similar proportions of the three nephron segments (Figure 2C). These experiments established a framework for using organoid plates to optimize and investigate differentiation conditions, revealing both dose-dependent and threshold effects.

**Marker Analysis Reveals Organoid Segments In Vitro**

Characterization of organoids using specific markers is important to determine which cell types are present and how they compare to tissues in vivo. In contrast to many organoid culture systems that require special processing, our organoid microwell

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**Figure 2. Utilization of Organoid HTS Plates to Optimize Differentiation**

(A) Representative well of a 384-well organoid plate (top row) robotically plated, differentiated, fixed, stained, and imaged for proximal tubule (LTL), distal tubule (ECAD), and podocyte (NPHS1) segments. Magenta overlay (bottom row) shows automatically identified structures over actual staining. Scale bar, 1 mm.

(B) Quantification of organoids/well in automated 384-well plates with increasing CHIR concentrations. Each box represents a single well. White boxes represent wells lost to fungal contamination. Z' factors for organoid differentiation in the three lines were calculated to be 0.596 (line 1), 0.034 (line 2), and 0.285 (line 3).

(C) Quantification of proximal tubules (green), distal tubules (yellow), and podocytes (red) at these different CHIR concentrations. Each condition shows the average of 32 wells (2 columns), and 14 μM shows the average of 64 wells. Conditions in which organoids did not differentiate efficiently (<5 organoids total) were not included in the analysis and appear blank.

See also Figure S2.

CHIR99021 has been applied at concentrations ranging from 5 to 12 μM in various kidney organoid differentiation protocols (Freedman et al., 2015; Lam et al., 2014; Mae et al., 2013; Morizane et al., 2015; Taguchi et al., 2014; Takasato et al., 2015), its dose dependence for organoid differentiation remains unclear. For the initial optimization, linear titrations of cell number were plated in 384-well kidney organoid plates and treated with three different concentrations of CHIR99021. On day 21 of differentiation, each well was fixed and stained for LTL and fully imaged using a high-content imager (15 min per plate). We then trained a computer to automatically identify and analyze individual organoids based on the presence of proximal tubules (Figure S2A). Differentiation was robustly achieved at all three CHIR99021 concentrations and cell numbers, with 3,000 cells/well and 14 μM CHIR99021 producing the optimal number of organoids per well (Figure S2B).
plates are adherent cultures that can be grown on glass, processed using standard techniques, and examined by confocal microscopy. We previously identified a variety of cell types within these cultures, including podocytes, proximal and distal tubules, endothelial cells, stromal myofibroblasts, and neurons (Cruz et al., 2017; Freedman et al., 2015; Kim et al., 2017). Using this approach, we further analyzed a panel of important markers whose localization patterns in kidney organoids remain poorly characterized, compared to human kidney tissues undergoing active nephrogenesis.

In both organoids and kidney tissues, proximal tubules exhibited strong LTL binding and weak ECAD expression, whereas distal tubules exhibited weak LTL binding and strong ECAD expression (Figures 3A, 3B, and S3A–S3C). In contrast to LTL, which was expressed in both proximal and distal tubules, expression of cubulin (CUBN), a receptor important for nutrient and protein reabsorption, was sharply restricted to proximal tubules, localizing strongly to the apical membrane (Figures 3A, 3B, and S3A–S3C). The cystic fibrosis transmembrane conductance regulator (CFTR) was detected in apical foci in both proximal and distal tubules, while the organic anion transporter (OAT1) localized to the basolateral membrane, similar to human kidney tissues in vivo (Figures 3A and 3B).

In the glomerular compartment, podocytes formed tightly clustered cell aggregates expressing NPHS1, synaptopodin, and WT1 (Figures 3A and 3B). We further investigated this compartment for parietal epithelial cells (PECs), an important cell type for kidney disease and regeneration (Shankland et al., 2017), which have not previously been identified in kidney organoids derived from hPSCs. In our multiwell organoids, we detected a population of cells adjacent to podocytes expressing CLDN1 and PAX8, markers that were absent in podocytes and were expressed by PECs in vivo (Figures 3A, 3B, and S3D). In a subpopulation of organoid structures, these CLDN1 “PAX8” cells surrounded the podocyte clusters in a lining one single-cell layer thick, which was positioned between the proximal tubular cells (LTL”) and the podocytes (podocalyxin”) but did not bind LTL (Figures 3C, 3D, S3E, and S3F). These structures and marker expression patterns were reminiscent of the PEC layer of Bowman’s capsule in vivo (Figures 3D and S3G).

In contrast to these proximal nephron segments, collecting ducts were not detected in organoids with two distinct segment-specific markers, Dolichus bifloris agglutinin (DBA), and aquaporin-2 (AQP2) (Figure 3E). These markers successfully distinguished ducts from tubules in developing kidney tissues (Figure 3E). These careful analyses of organoids versus tissues revealed more nuanced expression of nephron compartment markers than previously appreciated.

Enhancement of Endothelial Differentiation in HTS Organoids

The vasculature is a critical component of all somatic organs, with essential functions in physiology and disease. Organoid cultures derived from hPSCs lack mature vascular networks but can contain subpopulations of endothelial cells (ECs), which form linear chains of cells in contact with the organoids (Freedman et al., 2015; Takasato et al., 2015). As endothelial cells are typically a minor component in these cultures, we used kidney organoid microwells to optimize the differentiation protocol to increase ECs (Figure 4A). We discovered that addition of vascular endothelial growth factor (VEGF) during the differentiation process resulted in an approximately 10-fold increase in ECs expressing CD31 and vascular endothelial (VE)-cadherin, without compromising the formation of the organoids (Figures 4B–4E). These ECs resembled those obtained in an endothelial cell–directed differentiation protocol (Figure 4E) (Palpant et al., 2017). Despite their increased numbers, ECs did not invade the podocytes to establish a bona fide glomerular basement membrane (Figure 4F). Thus, increase in ECs alone was insufficient to induce glomerular formation in vitro, suggesting a requirement for additional cues such as specific extracellular matrix isoforms (Abrahamson et al., 2013), or a more specific endothelial cell population.

Single-Cell RNA Sequencing Reveals Spectrum of Organoid Maturation States

We further performed and analyzed single-cell RNA sequencing (scRNA-seq) to reveal gene expression signatures of individual cell types within our inherently heterogeneous organoids. Unsupervised clustering analysis of transcriptomes from 10,535 cells revealed a total of sixteen cell clusters, as visualized in t-distributed stochastic neighbor embedding (t-SNE) plots (Figures 5A and S4A–S4D). Six of these clusters were identified as kidney or endothelial cell lineages, based on comparison to gene lists of scRNA-seq clusters generated from developing human kidneys or newborn mouse kidneys (Figures 5A and 5B) (Adam et al., 2017; Menon et al., 2018). These included (1) proximal tubules, (2) podocytes, (3) “early tubules” expressing signatures of both proximal and distal tubules and collecting ducts, (4) “early podocytes” expressing signatures of both podocytes and PECs, (5) endothelial cells, and (6) stromal cells (Figures 5A and 5B). Collectively, these six clusters comprised ~60% of all cells analyzed by Drop-seq. The remaining ten clusters included neural, muscle, reproductive/endocrine, epithelial, undifferentiated, and proliferating cells, whereas a distinct population of collecting ducts was not identified (Figures S4A–S4D). Similarly, specific markers of collecting duct principal and intercalated cells were not detected in bulk RNA-seq analysis (Figure S4E).

Average gene expression within each of the six kidney-relevant organoid clusters correlated well with its corresponding kidney compartment in vivo (Figure 5C), based on comparison to scRNA-seq analysis of developing human kidneys (Menon et al., 2018). Notably, the endothelial cell cluster had a gene expression signature characteristic of ECs including PECAM1 (CD31), CD34, and KDR, and the overall gene expression of the cluster was clearly different from the stromal cell clusters (Figures 5A–5D). Relative to other cell clusters, an enhanced quantity of cells in the stromal cell cluster showed gene expression patterns characteristic of kidney interstitial myofibroblasts, pericytes, and mesangial cells (ACTA2, COL1A1, and TAGLN) (Brunskill et al., 2011; Daniel et al., 2012; Lin et al., 2008) (Figure 5D). While there was no distinct cell cluster for parietal cells, co-expression of CLDN1, PAX8, and NPHS1 within the early podocyte cell cluster suggested that it may contain developing PECs (Figure 5D).

As organoids included in this analysis were generated by treatment both with and without VEGF, we explored the contribution of cells to each cluster by each of the datasets (Figure 5E). Cells from each dataset were well dispersed within each cell cluster, confirming that organoids treated with VEGF generated a robust
Figure 3. Microwell Plates Reveal Detailed Patterning of Organoids Similar to Tissues In Vivo

A) Representative images of kidney organoids in microwell plates subjected to immunofluorescence analysis for segment-specific markers. Top row shows wide-field immunofluorescence image taken with a 4× objective. Middle row shows confocal image of the organoid highlighted above in the boxed region, taken with a 40× objective. Bottom row shows zoom of boxed region from middle row. ZO-1 (column 2) and CLDN1 (column 3) were labeled in the far red and red channels, respectively, in the same sample. Each of these is pseudocolored red and displayed separately to show co-localization with NPHS1 in the green.

B) 40× images (top) with zoom (bottom) of the same marker combinations in developing kidneys. Arrowheads (CFTR and CLDN1) indicate specific patterns in organoids and tissues.

C and D) Confocal images of organoids with progressive zooms, showing PEC-like expression of PAX8 (C) and CLDN1 (D) in LTL+ capsules surrounding podocytes (PODXL?), compared to human kidney tissue (D, right).

E) Confocal images of collecting duct markers, counterstained with LTL, in organoids and tissues. Scale bars, 100 μm. See also Figure S3.
fraction of epithelial and stromal cells on par with organoids without VEGF treatment (Figure 5E). Although VEGF clearly increased the number of ECs by immunofluorescence, relatively few ECs were captured by scRNA-seq and only a modest increase in ECs was observed (Figure 5E). In contrast, bulk RNA-seq analysis from replicate wells detected marked (4- to 12-fold) upregulation of endothelial cell markers including PECAM1, CD34, CDH5, and FLT1 after VEGF treatment, validating the immunofluorescence analyses (Figure S4F). The low abundance of ECs detected by scRNA-seq suggested either that a spectrum of maturation states was present in the cultures or that a substantial number of ECs were lost or destroyed during the processing steps prior to sequencing of Drop-seq isolates.

Subclustering of stromal cells further revealed a unique subpopulation, expressing the VEGF receptor FLT1, that arose specifically in VEGF-treated cultures but was entirely absent in untreated controls (Figures 5F–5I). MCAM, which was recently identified as a marker of endothelial cell progenitor cells within the developing kidneys (Halt et al., 2016), was strongly coexpressed within this subcluster (Figures 5F–5I). In the presence of VEGF, MCAM+ cells accounted for ~9.5% of cells within the six kidney clusters, or ~5% of all cells (Figure 5G). In VEGF-treated cultures, MCAM protein was specifically expressed in CD31+ cells occupying large portions of the surface area, consistent with the identification of these cells as endothelial cell progenitors (Figure 5J).

Endothelial cell-specific growth receptors were detected at low levels by scRNA-seq, despite substantial expression of their ligands from neighboring cells (Figure S4H). Although FLT1 could be clearly detected by bulk RNA-seq, a method that involves less processing and increased sampling, it was difficult to detect by scRNA-seq (Figures S4F and S4H). Collectively, these findings suggest that, while VEGF treatment greatly increases the number of endothelial cell progenitors in organoid cultures, only a small minority of these cells reaches a mature endothelial cell differentiation state similar to that found in vivo. Furthermore, a substantial number of ECs may be lost during the scRNA-seq processing steps.

Organoid Plates Model Kidney Injury and Disease

An important potential application for organoid-based microwell plates is to assess organ-specific toxicity and disease phenotypes using automated, HTS assays to predict safety and efficacy. In support of this approach, we first treated 384-well kidney organoid plates with increasing titrations of cis-diamineplatinum(II) dichloride (cisplatin), a chemotherapeutic with known nephrotoxic side effects (Freedman et al., 2015; Morizane et al., 2015; Pabla...
Using microscopy, we observed that cisplatin induced apoptosis and caused destruction to tubule organization in kidney organoids in a dose-dependent manner (Figure 6A and Video 2). This loss in cell viability could also be detected using a sensitive, luminescence-based assay appropriate for microwell formats (Figure 6B). To extend this analysis to specific biomarkers, which are more sensitive than toxicity, we measured kidney injury molecule-1 (KIM-1) expression using an ELISA-based approach and succeeded in detecting high levels of expression at sub-lethal doses (Figure 6C). Expression of KIM-1 specifically in the injured organoids was furthermore confirmed by immunofluorescence (Figure 6D).

We further investigated the potential of organoids in HTS formats to model genetic disease. Cyst formation is a common endpoint in many different kidney diseases, including the most common genetic cause of kidney failure, polycystic kidney...
disease (PKD). Gene-edited kidney organoids with mutations in polycystin-1 or polycystin-2, loss of which causes PKD, produced cysts from kidney tubules in automated, 384-well cultures (Figure 6E), similar to our findings in larger format wells (Freedman et al., 2015). To test the ability of organoids to respond physiologically to chemical stimuli, we treated them with forskolin, which induces swelling by activating chloride channels such as CFTR. Forskolin treatment resulted in cystic swelling of HTS kidney organoid tubules in a dose-dependent manner (Figures 6F and 6G) (Cruz et al., 2017). These assays established a technological framework for assessing the effect of chemical or genetic treatments on organoids, to distinguish true therapeutic efficacy from differentiation- or toxicity-induced false positives or false negatives in HTS experiments (Figure 6H).

Screening Reveals an Unexpected Role for Myosin in Organoid PKD

To test whether our HTS organoid platform could provide insights into disease, we performed a small-scale screen to identify modifiers of PKD. We focused on eight candidate factors that might modulate interaction of cells with their surrounding micro-environment, which we have recently discovered to be important in organoid PKD (Cruz et al., 2017). HTS organoids derived from gene-edited hPSCs with mutations in polycystin-1 were treated on the 21st day of differentiation, a time point at which cysts had not yet formed, and maintained in the presence of each compound for 7 days. In most of the treatment conditions, cyst formation generally ranged from 5% to 20% of organoids, with no compound showing a dose-dependent decrease in cystogenesis. Interestingly, however, blebbistatin, a specific inhibitor of non-muscle myosin II, or NMII (Straight et al., 2003), induced a significant increase in cyst formation at the highest concentration, 12.5 μM (Figure 7A). This was unexpected, as the myosin pathway is not known to be involved in PKD.

To validate this finding, we added blebbistatin to organoids in low-throughput suspension cultures, a condition that promotes robust cystogenesis from PKD organoids over the course of ~14 days (Cruz et al., 2017). In blebbistatin-treated suspension cultures, PKD organoids formed cysts after only 24 hr, which continued to increase dramatically in diameter over the next day.

Figure 6. Organoid HTS Plates Model Toxicity and Disease Phenotypes

(A–D) Individual organoids treated with increasing cisplatin doses showing (A) phase-contrast effects on tubular integrity, (B) quantification of cell survival, (C) KIM-1 expression detected by ELISA, and (D) KIM-1 immunofluorescence.

(E) Immunofluorescence images of a cyst formed in a 384-well plate from a kidney organoid with mutations disrupting the PKD2 gene.

(F) Phase-contrast images of organoids tubules with or without forskolin treatment.

(G) Quantification of cystogenesis induced by forskolin at increasing concentrations.

(H) Schematic of multi-dimensional data in HTS organoids. Each position represents a different treatment condition. A positive hit showing normal differentiation, low toxicity, and high efficacy (phenotypic rescue) is highlighted with an asterisk in the efficacy dataset.

Scale bars, 100 μm. Error bars, SD. *p < 0.05 (n = 3 or more experiments). See also Video S2.
week (Figure 7B). In contrast to nephrotoxic compounds such as cisplatin, we observed no damage to organoids with blebbistatin treatment at this concentration and time frame (Figure 7B). The rapid growth and expansion of blebbistatin-induced cysts suggested that they remained proliferative (Figure 7B), similar to PKD cysts without blebbistatin (Cruz et al., 2017). Blebbistatin increased the rate of cystogenesis ~40% in PKD organoids, but only ~10% in control organoids of identical genetic background that lacked PKD mutations (Figure 7C). Both the diameter and number of cysts were increased in blebbistatin-treated PKD organoids, compared to other conditions (Figure 7D). When blebbistatin was removed from these cultures, cyst size declined, indicating that blebbistatin’s effects were partially reversible and dependent upon sustained treatment with the drug (Figure 7E). Immunofluorescence analysis indicated that blebbistatin-induced cysts arose from proximal and distal tubular epithelial cells, but not from podocytes (Figures 7F and S5A). Non-muscle myosin IIB (NMIIb) was expressed much more strongly within these cyst-lining epithelial cells than in non-cystic LTL+ tubular structures located inside the same organoids (Figures 7G and S5B). Collectively, these findings suggested that inhibition of NMII promoted cystogenesis in PKD organoids.

**DISCUSSION**

Organoid cultures have significant advantages for HTS, including human species specificity, regenerative applications,
and the ability to model complex phenotypes, but have been limited to lineages with robust, self-renewing stem cells, such as intestinal crypt cells or mammary tumors (Gracz et al., 2015; Sachs et al., 2018). To our knowledge, hPSC-derived organoids have not previously been generated in automated, HTS-compatible formats. Although our work has focused on mini-kidney organoid differentiation as a representative lineage, it is likely that the same general techniques could be adapted to produce other types of organoids from hPSCs, such as mini-guts and mini-brains (Hayashi et al., 2016; McCracken et al., 2014; Spence et al., 2011). Importantly, all steps of differentiation, processing, imaging, and analysis can be performed automatically, using conventional cell-culture robots, or by hand.

Using this system, we have shown that hPSC-derived organoids in HTS formats can be experimentally manipulated to enhance and optimize their own differentiation. A threshold concentration of CHIR99021 is required to induce kidney lineage differentiation, above which organoids form with similar sub-compartment composition. Surprisingly, this induction threshold varies significantly between individual hPSC lines of identical genetic background. These organoids can be processed in multiplex fashion using low liquid volumes to identify previously unidentified cell types or subcompartments, such as PECs. scRNA-seq provides an unbiased mechanism to further assess our differentiation protocol, allowing us to confirm our immunostaining results and show that many of the cell types generated in our hPSC-derived organoids are indeed similar to those found in human kidneys during development.

One limitation of this work is that current organoid protocols produce fetal nephrons in which the tubules and vasculature are not fully mature (Freedman et al., 2015; Morizane et al., 2015; Taguchi et al., 2014; Takasato et al., 2015). This is most evident from our scRNA-seq analysis, which reveals significant clusters of early tubular cells and early podocytes, which are distinct from the more mature examples of these cell types. We demonstrate that the number of ECs can be greatly increased in organoids by VEGF supplementation, a step toward vascularization that may be generalizable to other types of organoids derived from hPSCs. Many of these ECs are not yet fully mature, similar to the epithelial cells within these organoid cultures. Future HTS screening, complemented with unbiased scRNA-seq, may identify additional factors that push these cells to mature into podocytes, proximal tubules, and ECs, to promote more sophisticated architectures such as glomerular basement membranes. Notably, vascularization alone is insufficient to produce fully functioning kidneys from developing metanephroi transplanted in vivo, underscoring the need for HTS optimization of maturation state and purity to produce functional, engraftable stem cell populations (Dekel et al., 2003; Harari-Steinberg et al., 2013).

The combination of hPSCs and HTS has great potential for reducing costs and increasing success rates of clinical drug development (Grskovic et al., 2011). Our work builds upon previous platforms in vitro using primary or hPSC-derived epithelial cells types for toxicity or disease modeling (Astashkina et al., 2012; Huang et al., 2015; Kandasamy et al., 2015; Ramm et al., 2016; Rinkевич et al., 2014). Our system is unique, however, in that it combines automated, HTS-compatible formats with hPSC-derived organoids, which are self-organizing, highly complex, and include cell types that are challenging to cultivate from primary cultures (Freedman et al., 2015; Morizane et al., 2015; Taguchi et al., 2014; Takasato et al., 2015). hPSC-derived organoids in HTS formats efficiently model tissue-specific toxicity and disease phenotypes, such as KIM-1 expression and cyst formation. In combination with careful analysis of differentiation on a well-to-well basis, these techniques will enable proper interpretation of library-scale drug discovery experiments and “clinical trials in a dish” in large cohorts representing diverse patient populations (Doulatov et al., 2017; Huang et al., 2015; Sachs et al., 2018; Yang et al., 2013).

Despite many years of research, the molecular functions of the polycystin proteins remain enigmatic, which has hampered the development of targeted therapies for PKD. Applying our HTS platform, we have identified blebbistatin, an inhibitor of NMII ATPase activity, as a specific activator of PKD cystogenesis in organoids. This suggests that the polycystins may normally function to positively regulate actomyosin activation within the tubular epithelium, strengthening and tightening the tubule and preventing it from deforming into a cyst. Actomyosin is known to play important roles in epithelial cell-cell adhesion at adherens junctions, tight junctions, and focal adhesions, including the formation of purse-string-like contractile rings (Conti et al., 2004; Vicente-Manzanares et al., 2009). During normal kidney development, myosin similarly promotes the proper elongation of nephron tubules (Lienkamp et al., 2012). In addition to epithelial cells, stromal myofibroblasts could also be affected by blebbistatin, as these constitute a significant subpopulation within organoids by scRNA-seq, and can be associated with PKD cysts (Cruz et al., 2017). Interestingly, recent work has identified a possible myosin heavy chain-like, calmodulin-binding domain at the carboxyl terminus of polycystin-1, which could potentially regulate myosin (Doen et al., 2016). The precise mechanism whereby myosin and PKD pathways interact is an interesting area for future investigation, which may provide directions for therapy development. HTS organoids thus provide a screening tool to catalyze discoveries, which can be further evaluated in lower-throughput systems in vivo such as mouse models and human clinical trials.

In conclusion, we have produced organoid plates from hPSCs in microwell formats capable of modeling complex human differentiation and disease states. These organoid plates bridge the gap between HTS-compatible experimental models, such as 2D cell lines and recombinant proteins, and low-throughput models, such as rodents. This balance of complexity and high throughput, coupled with their inherent species specificity, provides an attractive starting point for screening approaches focusing on therapeutic discovery, toxicology, and regenerative medicine.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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QUANTIFICATION AND STATISTICAL ANALYSIS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two videos and can be found with this article online at https://doi.org/10.1016/j.stem.2018.04.022.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS


REFERENCES


Attayek, P.J., Balowski, J., Liu, X.F., Laurenza, R.J., et al. (2015). A high-throughput Core, Ellisson Stem Cell Core, Garvey Imaging Core (UW ISCRM), and DNA Sequencing Core (UM).


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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Benjamin Freedman (benof@uw.edu).
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human studies were performed with informed consent under the auspices of the University of Washington IRB. Studies with human pluripotent stem cells were performed with approval by the University of Washington ESCRO. WA09 (H9) female embryonic stem cells (WiCell) or WTC11 iPSCs derived from a Japanese male donor (gift of Dr. Bruce Conklin, Gladstone Institute) were maintained in 6-well tissue-culture treated dishes (Falcon) at 37 degrees feeder-free on 1% Reduced Growth Factor GelTrex (Life Technologies) in 2mL mTeSR1 (Stem Cell Technologies). Experiments in mice were performed in compliance with the strict ethical requirements and regulations of the UW IACUC under a pre-approved animal protocol. A colony of NOD.CB17-Prkdcscid/J mice (NOD-scid, Jackson Laboratory) was maintained under specific pathogen free conditions. Littermate animals of equally mixed genders and 6 weeks of age were used for all experiments.

METHOD DETAILS

Kidney differentiation in microwell plates

hPSCs were dissociated with Accutase (Stem Cell Technologies) and plated onto microwell plates pre-coated with GelTrex in mTeSR1 supplemented with 10 μM Rho-kinase inhibitor Y27632 (StemGent). The media was replaced with mTeSR1 + 1.5% GelTrex at 16 hours, 12 μM CHIR99021 in Advanced RPMI + Glutamax (Life Technologies) at 60 hours, and RB (Advanced RPMI + Glutamax + B27 Supplement, from Life Technologies) at 96 hours. Volumes used are as follows: 500 μL for 24-well plates, 100 μL for 96-well plates, and 50 μL for 384-well plates. RB was changed two days later and every three days thereafter. For experiments involving modulation of endothelial cells, the media was supplemented with VEGF165 (Peprotech, 12.5 to 200 ng/ml). Alternatively (Protocol B, Figure S1A), the protocol described by Takasato et al. was adapted for adherent culture: undifferentiated hPSCs were plated overnight and treated the following morning with 8 μM CHIR99021 in APEL media (StemCell Technologies) for 48–72 hr, 30 ng/ml FGFR (Peprotech) + 1 μg/ml heparin (StemCellTechnologies) in APEL for 96 hr, and cultured thereafter in APEL, replaced every three days. Alternatively, to generate endothelial cells without kidney organoids, 100,000 hPSCs/cm² were plated in mTeSR1 + 10 μM Y27632 + 1 μM CHIR99021, replaced with RPMI + B27 minus insulin + 1.5% Geltrix + 50 ng/mL Activin A (R&D) at 24h, RPMI + B27 minus insulin + 40 ng/mL BMP4 (Peprotech) + 1 μL CHIR99021 at 61 h, and StemPro 34 (Thermo Fisher Scientific) + 2 mM Glutamax + 50 μg/mL ascorbic acid (Sigma) + 10 ng/mL BMP4 + 5 ng/mL bFGF (Peprotech) + 300 ng/mL VEGF165 at 85 h for a 72-hour incubation. Robotic instrumentation consisted of a BioTek EL406 plate washer with microplate stacker from Beckman-Coulter Matrix Technologies, WellMate Dispenser and Stacker and a CyBio CyBi-Well Vario Workstation which allows dispensing of small amounts of reagents, cells, and compounds. Manual instrumentation consisted of Integra Voyager and Viaflo II electronic multichannel pipets.

Teratoma formation

Dissociated hPSCs (400,000/well) were plated in three wells of a 6-well plate and grown to confluence in mTeSR1 for six days. Cells were dissociated, pelleted, resuspended in 500 μl of an ice-cold 1:1 mixture of DMEM/F12 (Fisher) and Matrigel (Corning). The cells were immediately injected beneath the neck scruff of immunodeficient, NOD-scid mice using a 22-gauge syringe needle. Growth were harvested 15 weeks after injection, photographed, fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid, all from Sigma), embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological analysis.

Immunohistochemistry

For confocal microscopy, kidney organoids were differentiated on 96-well No. 1.5 coverslip glass-bottom plates (Mat-Tek). To fix, an equal volume of 8% paraformaldehyde (Electro Microscopy Sciences) was added to the culture media (4% final concentration) for 15 minutes at room temperature. After fixing, samples were washed in PBS, blocked in 5% donkey serum (Millipore)/0.3% Triton X-100/PBS, incubated overnight in 3% bovine serum albumin/PBS with primary antibodies, washed, incubated overnight with Alexa Fluor secondary antibodies and DAPI (Invitrogen), and washed in PBS. Primary antibodies included ZO-1 (339100; Invitrogen), PAX8 (10336-1-AP, Proteintech), NPHS1 (AF4269, R&D), OAT1 (PA6-26244, Thermo Fisher), CLDN-1 (sc-21537; Santa Cruz), E-CAD (ab11512, Abcam), WT1 (sc-192; Santa Cruz), CFTR (570 antibody; University of North Carolina), Myosin IIB (3404S, Cell Signaling), CUBN (gift of Dr. Dennis Brown, Massachusetts General Hospital), AQP2 (HPA046834, Sigma), CD114/VE-cadherin (2500T, Cell Signaling), CD146/MCAM (ab75769, Abcam), and CD31/PECAM (555444; BD). LTL (FL-1321, Vector Laboratories) and DBA (B-1035, Vector Laboratories) were similarly applied. Fluorescence images were captured using an inverted Nikon epifluorescence Eclipse Ti or A1R confocal microscope. Automated imaging was performed using a GE INCELL 2000 Analyzer.

Automated organoid optimization and analysis

Organoids were produced in a fully-automated manner and developed to an age of 25 days, then fixed and stained with NPHS1, LTL, and ECAD to mark podocytes, proximal tubules, and distal tubules respectively. Each well was imaged at a standardized exposure using an In Cell Analyzer 2000 (GE Healthcare). Representative images were collected using the GE INCELL investigator suite. An algorithm was then generated using the INCELL developer suite to accurately identify each population of cells while simultaneously excluding background fluorescence. We used this algorithm to count and measure the cell populations in each well, as well as across dosages of CHIR99021. These results were displayed using the Spotfire software (TIBCO) with the definition of an organoid as being a...
discrete group of cells that contains overlapping staining for podocytes, proximal tubule and distal tubule. To assess nephrotoxicity, organoids were purified manually and subjected to a dose titration of cisplatin (Sigma) for 24 hours in 96-well plates. Organoids were imaged and then fixed for immunofluorescence, or alternatively lysed and a KIM-1 ELISA (MesoScale Discovery) was performed. Organoid viability was assessed with CellTiter-Glo (Promega) and quantified using a PerkinElmer Envision plate reader.

**Cyst generation**

*PKD1*<sup>−/−</sup> and *PKD2*<sup>−/−</sup> hPSCs or isogenic controls (all generated previously by our lab) were differentiated in microwell plates in adherent cultures. Forskolin (LC Laboratories) was added to microwell plates during automated liquid handling on the 21<sup>st</sup> day after differentiation. Large swellings rapidly developed and grew to full size over 72 hours. Cysts were identified by comparing images captured with high-content imaging prior to forskolin treatment, and after 72 hours. Screening of PKD cystogenesis was performed in duplicate in 96-well plates to provide sufficient space and numbers of organoids per well. Factors were plated at four different concentrations for seven days, and the organoids were scanned visually on a phase-contrast microscope for increased or decreased cyst formation. Factors included blebbistatin (Cayman Chemicals, used at 0.1 μM, 0.5 μM, 2.5 μM, 12.5 μM), gelatin (StemCell Technologies, used at 0.1 μM, 0.5 μM, 2.5 μM, 12.5 μM), collagenase type IV (StemCell Technologies, used at 0.1 μM, 0.5 μM, 2.5 μM, 12.5 μM), GM 6001 (Cayman Chemicals, used at 0.1 μM, 0.5 μM, 2.5 μM, 12.5 μM), synthetic peptide derived from Vitronection (kindly provided by Cole DeForest at UW Chemical Engineering, used at 10 μM, 50 μM, 250 μM, 1.25 mM), synthetic peptide derived from bone sialoglycoprotein (kindly provided by Cole DeForest at UW Chemical Engineering, used at 10 μM, 50 μM, 250 μM, 1.25 mM), synthetic RGD peptide (kindly provided by Cole DeForest at UW Chemical Engineering, used at 10 μM, 50 μM, 250 μM, 1.25 mM), rMMP8, human (kindly provided by Cole DeForest at UW Chemical Engineering, used at 30 μg/ml, 6 μg/ml, 1.2 μg/ml, 0.24 μg/ml). To test cystogenesis in suspension, adherent organoids were microdissected with a 23-gauge syringe needle from 24-well plates on an inverted phase-contrast microscope, and transferred into a low-adhesion 6-well plate (Corning) containing 2 mL RB or 2 mL RB with 12.5 μM blebbistatin. Organoids were imaged daily on a Nikon Ti Inverted Widefield microscope for a period of 7 days. Cyst diameters were measured using NIS Elements imaging software (Nikon). Contiguous microscopic fields were collected using an automated stage and stitched together using NIS Elements software to generate large images of wells or plates.

**scRNA-seq and cell clustering analysis**

Organoids were collected by scraping cells from whole wells into ice-cold DPBS, dissociated with cold active protease (Adam et al., 2017), and Drop-seq was performed as on an Illumina HiSeq 2500 in rapid run mode. Sequences were aligned to NCBI human genome assembly GRCh38, with 70%–80% overall alignment. Organoid differentiation was performed from WA09 hPSCs (WiCell, Madison WI) in 24-well plates to provide sufficient space for analysis. Unsupervised cell clustering, principal components analysis and data presentation were performed with the following modifications/specifies: datasets from Drop-seq analyses of individual wells were combined and batch corrected. Unsupervised subclustering was performed following supervised selection of stromal cells from the initial clustering analysis. Cells were excluded if genes expressed were < 500 or ≥ 4000 (to exclude cell doublets) or if mitochondrial gene expression was > 25% all genes (to exclude non-healthy cells). Publicly available data were used for cell type identification and gene expression comparison: GUDMAP (https://www.gudmap.org/), GenePaint (https://www.genepaint.org), ESBK’s Kidney Systems Biology Project’s transcriptomic data (https://hpcwebapps.cit.nih.gov/ESBK/Database/), Gene Expression Omnibus accession number GSE94333, and KeyGenes (http://www.keygenes.nl/). A correlation matrix comparing average gene expression in organoid and human kidney clusters was generated using Stats R-package, based on GSE94333. Unless otherwise noted, “top differentially expressed genes” were chosen from top 20 for each organoid cell cluster, based on their appearance in scRNA-seq data from human developing kidney (corresponding clusters) and mouse P1 kidney (clusters of same lineage). Genes were listed in order of statistical significance with highest *p*-value for last gene generated based on number of cells in the cluster. scRNA-seq samples were deposited in the Gene Expression Omnibus (NCBI) under accession number GSE94333.

**Bulk RNA sequencing**

Cells from organoid cultures grown in parallel for scRNA-seq (+ and - VEGF treatment) were lysed with TRIzol Reagent (Invitrogen). Total RNA was isolated using Direct-zol mini prep RNA columns (Zymo Research) with on-column DNase treatment. RNA quality was assessed by Bioanalyzer 2100 platform (Agilent) using a Eukaryote Total RNA Nano array (Agilent), with RIN values of 9.7 and 9.5 for VEGF + and - samples, respectively. cDNA was generated from 10 ng RNA using the SMART-Seq Low Input RNA kit for sequencing (Takara) applying 8 PCR cycles. Amplified cDNA was purified by Agencourt AMPure XP DNA purification kit (Beckman Coulter) and analyzed on the Bioanalyzer platform using a High Sensitivity DNA array (Agilent). Next-generation sequencing libraries were generated and barcoded using the Nextera XT DNA Library Preparation Kit (Illumina) starting with 100 pg cDNA. cDNA libraries were pooled and sequenced on one lane of a HiSeq500 platform with Illumina TruSeq v4 chemistry (paired-end 2x75 cycles) at the University of Michigan DNA Sequencing Core. Resulting sequences were aligned to human genome (Ensembl GRCh38) using STAR (version 2.5.2) with default parameters. Relative read counts at gene level were estimated using HTP (version 2.20.2 and normalized using quantile normalization function in edgeR R statistical package. A total number of 52 and 76 million reads were obtained with alignment rates of 91% and 88% from VEGF + and − samples, respectively.
QUANTIFICATION AND STATISTICAL ANALYSIS

Data summaries shown in the figures are representative of three or more separate experiments (biological replicates). Statistical significance was calculated with Graphpad Prism software. For comparisons between two groups, a two-tailed Student’s t test for samples with unequal variance (heteroscedastic) was utilized. For comparisons between multiple groups, the analysis of variance (ANOVA) method was used. Z’ factor was calculated based on standard deviation as described (Zhang et al., 1999) to assess high throughput assay quality. The Z’ factor calculation provides a quantitative measure of the separation between our control (no CHIR99021) condition and the optimal differentiation condition for each line. A Z’ factor > 0.5 indicates an excellent assay (Zhang et al., 1999).

DATA AND SOFTWARE AVAILABILITY

The accession number for the scRNA-seq samples reported in this paper is GEO: GSE109718. CHIR99021 titration data are provided in Mendeley Data (https://doi.org/10.17632/988tyf4fh8.1).