Highly reproducible and cost-effective one-pot organoid differentiation using a novel platform based on PF-127 triggered spheroid assembly

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Highly reproducible and cost-effective one-pot organoid differentiation using a novel platform based on PF-127 triggered spheroid assembly

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Abstract
Organoid technology offers sophisticated in vitro human models for basic research and drug development. However, low batch-to-batch reproducibility and high cost due to laborious procedures and materials prevent organoid culture standardization for automation and high-throughput applications. Here, using a novel platform based on the findings that Pluronic F-127 (PF-127) could trigger highly uniform spheroid assembly through a mechanism different from plate coating, we develop a one-pot organoid differentiation strategy. Using our strategy, we successfully generate cortical, nephron, hepatic, and lung organoids with improved reproducibility compared to previous methods while reducing the original costs by 80%–95%. In addition, we adapt our platform to microfluidic chips allowing automated culture. We showcase that our platform can be applied to tissue-specific screening, such as drug toxicity and transfection reagents testing. Finally, we generate NEAT1 knockout tissue-specific organoids and show NEAT1 modulates multiple signaling pathways fine-tuning the differentiation of nephron and hepatic organoids and suppresses immune responses in cortical organoids. In summary, our strategy provides a powerful platform for advancing organoid research and studying human development and diseases.

1. Introduction
The study of human organ development and diseases is limited by the shortage of human tissue samples due to practical and ethical issues. Fortunately, numerous in vitro cell culture models have been established through the years, which have offered tremendous insights for the mechanistic understanding of human development and diseases. In particular, the derivation of human embryonic stem cells (hESCs) in 1998 have made it possible to partially mimic human organ development in vitro, owing to their infinite self-renewal ability and potential to differentiate to almost any tissues of human body [1]. In 2007, human induced pluripotent stem cells (iPSCs) were successfully obtained by the reprogramming of somatic cells, which are equivalent to hESCs in terms of their self-renewal ability and differentiation potential [2]. Moreover, iPSCs can be generated by reprogramming cells derived from patients, therefore allowing personalized disease modeling and drug discovery. In early days, differentiation of hESCs or iPSCs is usually carried out in two-dimensional (2D) monolayer culture. However, 2D culture lacks the
essential features of in vivo tissue environment such as cell polarity and organization structure, therefore cannot mimic the self-organization pattern or function of human organs. Fortunately, the development of three-dimensional (3D) culture system has partially filled this gap in recent years [3].

In 1987, a landmark study from Bissel group demonstrated that 3D ducts and lumen can be formed by breast epithelia grown on Matrigel, a heterogeneous and gelatinous protein mixture secreted by Engelbreth–Holm–Swarm mouse sarcoma cells [4]. More interestingly, the 3D cultures secret milk, a property that is expected of breast epithelia but lacked in 2D culture, highlighting the suitability of 3D culture in studying in vivo tissue development and function. These structures represent early examples of organ-like structures which are later named as organoids. In 2008, Sasai and colleagues generated cortical tissues containing distinct zones from mouse and human ESCs using a 3D aggregation culture method, demonstrating that organogenesis can be recapitulated using ESCs and 3D culture [5]. In 2009, Clevers and colleagues generated intestine organoids with crypt-villus structures from a single Lgr5+ adult intestinal stem cells in Matrigel [6]. These pioneer studies kick off the field of organoid research. Currently, organoids of various tissues have been established from ESCs or adult stem cells, including brain [7–13], gut [6, 14–17], stomach [18–22], liver [23–28], kidney [29–34], and lung [35–40]. Through the years, organoids have been proven broadly useful in applications such as disease modeling [7, 13, 25, 34, 41–44], drug testing [30, 34, 43–47], biobanking [48–52], and human development studies [17, 22, 29, 30, 35, 53]. In addition, coupled with genome editing technology, organoids have been expanded to novel applications such as studying brain evolution and traits linked to human cognition [54].

Many different methods have been developed for the production and culture of organoids, including extracellular matrix (ECM) scaffold, bioreactor, hanging drop, and low-adherent culture plate methods [55]. These methods often contain complicated multistep procedures and have associated shortcomings such as high cost, laborious media changing procedure, and low reproducibility. To make this more complicated, some protocols for liver and lung organoids start with differentiation of progenitor cells in 2D culture followed by dissociation and ECM scaffold-mediated assembly of 3D organoids [28, 36]. Furthermore, most organoids are cultured in or on Matrigel, an expensive and poorly defined animal product that displays batch-to-batch variation in quality and composition and could trigger potential immune adverse reactions due to the presence of mouse xenografts [56]. The replacement of Matrigel by purified ECM components [57] or synthetic hydrogels [58] facilitates the setup of more defined and reproducible environment, but these replacements fail to fully recapitulate the natural environment of tissues and do not provide necessary signals and factors for cell differentiation [59]. In addition, hydrogels derived from decellularized tissues contain unknown factors and are limited by tissue sources and ethics constraint [59]. Finally, the usage of hydrogels makes it difficult for downstream cell and imaging analysis. Taken together, the consistency, uniformity, and reproducibility remain to be the major challenge for organoid research [60].

To address some of the limitations, such as lacking of uniformity, microfabricated and microfluidic devices have been constructed to produce size-controlled culture area for uniform organoids culturing [61–64]. In addition, 3D bioprinting method has been proposed to make cell-bioinks at equal volume for organoids generation with size uniformity [65].

Pluronic, a type of non-ionic surfactant [66], which belongs to the class of amphiphilic triblock copolymers consisting of a central poly(propyleneoxide) block surrounded by two poly(ethyleneoxide) blocks [67], has been broadly used to block cell adhesion on polydimethylsiloxane (PDMS) surface [68]. In the recent years, Pluronic has been used by coating on PDMS chips to assist spheroid and organoid culture. The combination of Pluronic F-68 and bovine serum albumin (BSA) pretreatment on PDMS surfaces has been shown to enhance surface morphology, leading to optimized surfaces with anti-fouling properties, thereby promoting uniform production of spheroids from breast cancer cell line MDA-MB-231 [69]. Pre-coating PDMS microchips with Pluronic F-108 has been demonstrated to facilitate the generation of human ovarian cancer spheroids [70]. Pluronic F-127 pre-coating on PDMS microwells or chips has also been widely employed in the culture of various types of spheroids, including those formed by spermatogonial stem cells [71] and various types of cancer cells [72–75].

In the present study, we develop a relatively standardized procedure for organoid production (figure 1) by making several modifications over previous organoid culturing methods. First, we invented a spheroid assembly method that produces highly uniform hESC spheroids by including low concentration of PF-127 in culture media in untreated U-shape 96-well plate. The use of PF-127 and untreated U-shape 96-well plate significantly lowers the cost of organoid culture. Second, by treating hESC spheroids with appropriate differentiation media, we successfully generated organoids of tissues from all three germ layers including cortical, nephron, hepatic, and lung. Consistently, these organoids show high uniformity and batch-to-batch reproducibility. Third, we designed a microfluidic chip culture system to adapt our protocol that allows semi-automatic
culture of different organoids. Finally, we successfully applied our platform for drug toxicity testing, transfection reagent screening, and tissue-specific gene function analysis. Taken together, we have successfully designed a relatively standardized strategy for easy to operate, scalable, and reproducible stem cell organoid culture, which may provide a powerful platform for advancing organoid research and related applications in studying human development and diseases.

2. Materials and methods

2.1. Cell culture and assembly of hESC spheroids

The WIBR3 hESCs (NIHhESC-10-0079) were from Whitehead Institute. The H9 hESCs (WA09) were from WiCell. The V6.5 mESCs are the same cell line from a previous study [76]. WIBR3 and H9 hESCs were cultured in mT eSR1 medium (STEMCELL Technologies) on Matrigel (Corning) coated plate with daily medium change. hESCs were passaged as single cells using Accutase (Gibco) typically at ~80% confluency. V6.5 mESCs were cultured in serum medium as previously described [77] on gelatin-coated plates with daily medium change. All the cells were cultured at 37 °C in a 5% CO₂ humidified incubator. For spheroid formation, around 5,000 hESCs were seeded in mTeSR1 medium with 50 µM ROCK inhibitor Y-27632 (Selleck) and 0.0125% PF-127 (Sigma) per well in a 96-well non-treated round bottom microplate (Corning). No centrifugation was needed for spheroid formation. After ~24 h, 3D hESC spheroids were formed and processed for analyses or further differentiation.

2.2. Construction of hepatic, nephron, cortical, and lung organoids

To generate hepatic organoids, 3D hESC spheroids were treated with RPMI 1640 (Gibco) supplemented with 1 × B27 without insulin (Gibco) and 100 ng ml⁻¹ Activin A (R&D) to induce definitive endoderm differentiation at day 0 for six days. At day 6, the medium was replaced with RPMI 1640 containing 1 × B27 (Gibco), 10 ng ml⁻¹ bFGF (PeproTech), and 20 ng ml⁻¹ BMP4 (R&D) to induce hepatic endoderm differentiation. At day 9, the medium was switched to hepatocyte culture medium (HCM, Lonza), which consists of 5% FBS (PAN), 20 ng ml⁻¹ Oncostatin M (R&D), 100 nM dexamethasone (Sigma), and 10 ng ml⁻¹ HGF (MedChemExpress). The hepatic differentiation protocol was adapted from previous studies [78, 79].

To generate nephron organoids, 3D hESC spheroids were treated with basal medium consisting of Advanced RPMI 1640 (Gibco) supplemented with 100 × GlutaMAX supplement (Gibco), 10 µM CHIR99021 (Sigma), and 500 nM dorsomorphin (Tocris) at day 0 for four days. At day 4, the medium was replaced with basal medium containing 10 ng ml⁻¹ Activin A. At day 7, the medium was replaced with basal medium consisting of 10 ng ml⁻¹ FGFind (R&D). At day 9, the medium was replaced with basal medium consisting of 10 ng ml⁻¹ FGFind and 3 µM CHIR99021. At day 11, the medium was changed to the basal medium supplemented with 10 ng ml⁻¹ FGFind. At day 14, the medium was switched to basal medium. The nephron differentiation protocol was adapted from a previous study [30].

To generate cortical organoids, 3D hESC spheroids were treated with KSR medium containing KnockOut DMEM (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 100× GlutaMAX supplement, 0.1 mM nonessential amino acids (Gibco), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin, and 20% KnockOut Serum Replacement (Gibco) supplemented with 2.5 µM dorsomorphin and 10 µM SB431542 (Tocris) at day 0 for six days. At day 6, the medium was changed to neurobasal medium containing Neurobasal A (Gibco), 1 × B27 without
differentiation protocol was adapted from a previous study [9]. To generate lung organoids, 3D hESC spheroids were treated with RPMI 1640 supplemented with 100 ng ml\(^{-1}\) Activin A at day 0 for one day. At day 1, the medium was replaced with RPMI 1640 supplemented with 100 ng ml\(^{-1}\) Activin A and 0.2% FBS. At day 2, the medium was replaced with RPMI 1640 supplemented with 100 ng ml\(^{-1}\) Activin A and 2% FBS. At day 4, the medium was changed to N2B27 medium containing DMEM/F12 (Gibco), 1 × N2 (Gibco), 1 × B27, 100 × GlutaMAX supplemented with 10 µM SB431542, 100 ng ml\(^{-1}\) Noggin (Novoprotein), 1 µM SAG (Selleck), 500 ng ml\(^{-1}\) FGF4 (Novoprotein), and 2 µM CHIR99021. At day 9, the medium was switched to N2B27 medium supplemented with 500 ng ml\(^{-1}\) FGF10 (Novoprotein) and 1% FBS. The lung differentiation protocol was adapted from a previous study [36].

2.3. RNA extraction and qRT-PCR analysis
Total RNA was extracted using TRIzol Reagent (Magen) and reverse transcribed into cDNA using the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme) following the manufacturer’s instruction. qRT-PCR was performed using Realtime PCR Super mix SYBR green (Vazyme) on StepOne Plus Real-Time PCR System (Applied Biosystems). The data were normalized to the level of β-ACTIN in each sample. Primers for qPCR are listed in supplementary table 12.

2.4. Immunofluorescence (IF) staining
Organoids were first washed with phosphate buffered saline (PBS) three times and then fixed in 4% paraformaldehyde (Leagene) for 30 min at room temperature. After rinsing three times with PBS, organoids were then incubated in 30% sucrose at 4 °C overnight. Organoids were embedded in tissue freezing medium optimal cutting temperature (OCT) compound (SAKURA) and cryo-sectioned by a cryostat (Leica). After washing with PBS three times, sections were permeabilized with 0.1% Triton-X in PBS for 15 min and then incubated in blocking medium (5% FBS and 0.1% Triton-X in PBS) for 1 h at room temperature. After blocking, sections were covered with primary antibodies diluted in blocking medium at 4 °C overnight. Sections were then washed three times with PBS and incubated with secondary antibodies diluted in PBS in the dark at room temperature for 4 h. Primary and secondary antibodies were listed in supplementary table 13. Images were obtained using fluorescence microscope (Nikon), an Andor spinning disk confocal microscope (Andor), or Leica confocal microscope (Leica) and analyzed and processed with Fiji for Mac OS X.

2.5. Functional assays of hepatic and nephron organoids
For lipid storage analysis, hepatic organoids were cryo-sectioned and treated with Oil Red O kit (Abcam) following the manufacturer’s instruction. To evaluate glycogen storage, fresh hepatic organoids were cryo-sectioned omitting sucrose incubation step and stained by periodic acid-Schiff (Solarbio) according to the manufacturer’s instruction. For indocyanine green (ICG) uptake and release, organoids were incubated with 1 mg ml\(^{-1}\) of ICG for 20 min at 37 °C in 5% CO\(_2\). Images of ICG uptake were taken under a microscope, then the organoids were gently washed 3 times with PBS, and fresh medium was added. After 6 h of incubation at 37 °C in 5% CO\(_2\), images of ICG release were taken under a microscope. Levels of albumin and alpha-fetoprotein secretion of hepatic organoids were determined by ELISA. The supernatant was collected after 24 h incubation with hepatic organoid. Human albumin and α-fetoprotein were determined using the Human Albumin and AFP ELISA kits (Abcam) according to the manufacturer’s instructions. The production level of urea in the medium was determined by using the QuantChrom urea assay kit (BioAssay Systems). For dextran uptake assay, nephron organoids were incubated with Alexa Flour 488 (AF488) conjugated dextran (Invitrogen) following the manufacturer’s instruction.

2.6. Toxicity test of antibiotics
Erythromycin (MedChemExpress), roxithromycin (Pharmabiology), azithromycin (Pharmabiology) and cefepime (Pharmabiology) stock solution were prepared in DMSO. Streptomycin (Biomed), gentamycin (Pharmabiology), neomycin (Pharmabiology), sodium cephalosporine (Pharmabiology), and penicillin (Pharmabiology) stock solution were prepared in DMSO. Ciprofloxacin (Coolaber), ofloxacin (Pharmabiology) and norfloxacin (Pharmabiology) stock solution were dissolved in RPMI 1640 medium. Ciprofloxacin (Coolaber), ofloxacin (Pharmabiology) and norfloxacin (Pharmabiology) stock solution were dissolved in RPMI 1640 medium containing 0.1 mol l\(^{-1}\) hydrochloric acid. The stock solutions were diluted with corresponding medium at indicated concentrations and then used to culture organoids. The drug treatment was initiated at day 9 or day 10 for hepatic, nephron, and lung organoids and day 25 for cortical organoids and lasted for 3 d before assessing viability.

2.7. Viability assessment of organoids
The viability of organoids following drug treatment was assessed by measuring ATP level using CellTiter-Glo® 3D Cell Viability Assay (Promega) according to the manufacturer’s instruction. CellTiter-Glo® 3D reagent was added to organoid culturing plate...
in the dark at room temperature. The plate was shaken at 400 r.p.m. for 5 min and then stand for 25 min. Luminescence measurement was performed in a white opaque 96-well plate by a microplate reader (Berthold).

2.8. Microfluidic chip fabrication

Microfluidic chips were made of PDMS and fabricated by soft lithography procedure. The PDMS chip had two layers. The top layer was molded by PDMS pre-polymer containing a 5:1 (w/w) mixture of the PDMS precursor and curing agent (Momentive). And the bottom layer was molded by 10:1 (w/w) mixture of the PDMS precursor and curing agent. The top layer consisted of medium channels to transport differentiation medium and the bottom layer contained 3 mm square pyramids to culture organoids. The pre-polymer in the channels was then polymerized by thermal curing in an 80 °C dry oven for 1 h. The top layer of the chambers was adhered to the bottom layer after oxygen plasma treatment.

2.9. Generation of hESC derived spheroids and organoids on microfluidic chip

Two-layer PDMS chips were sterilized by washing with 75% ethanol three times and exposing to 254 nm ultraviolet light for 30 min. The chips were then pretreated with 1.25% PF-127 for 30 min at room temperature. After removing 1.25% PF-127, around 2.10 mL of medium per well. Another 50 µl media were added to each sample of medium channel and a disposal bottle. Hepatic and cortical organoids on chips were differentiated and cultured with a continuous flow of corresponding medium at a rate of ~60 µl per day per well using a syringe pump (Leadfluid).

2.10. mRNA-LNP formation

The mRNA-lipid nanoparticle (mRNA-LNP) formulations were prepared as previously described [80]. Briefly, luciferase (Luc) mRNA was dissolved in citrate buffer (10 mM, pH 4.0) and lipids were dissolved in ethanol. Above solutions were mixed at a ratio of 3:1 (mRNA: lipids, vol: vol) to reach the final weight ratio of 20:1 (total lipids: mRNA). After 10 min incubation at room temperature, the formulation was diluted with 1 × PBS to meet the transfection needs. To test and compare transfection efficacy of mRNA-LNP in multiple organoids, three kinds of LNPs consisted of different lipid components were prepared (mol/mol ratio):

- LNP-1: SM102/DSPC/cholesterol/DMG-PEG/DOTAP equal to 50/10/38.5/1.5/42.9;
- LNP-2: SM102/DSPC/cholesterol/DMG-PEG/DOTAP equal to 50/10/38.5/1.5;
- LNP-3: SM102/DSPC/cholesterol/DMG-PEG/18PA equal to 50/10/38.5/1.5/11.1;
- Lipo3000 (Invitrogen) was used as a control.

2.11. Organoid transfection

Briefly, individual hepatic, nephron, lung, and cortical organoids at day 12 were transfected with the mass of 1 µg luciferase mRNA with LNP-1, LNP-2, LNP-3, and Lipo3000 (Invitrogen). To test selectivity of transfection reagents against different organoids, four types of organoids at day 12 were transferred to one well of non-treated round 96-well microplate and then transfected with 1 µg luciferase mRNA by LNP-1, LNP-2, LNP-3, and Lipo3000. After 24 h, organoids were lysed using Lysis 5× Reagent (Promega). The luciferase expression was measured according to the manufacturer’s instruction (Promega).

2.12. NEAT1 knock out

Guide RNA (gRNA) sequences were designed on the website http://crispr.mit.edu/ to target NEAT1 promoter and gene body. Two gRNA sequences were 5′-CGGGCACCTCCTTTGCTAGG-3′ and 5′-AACGTTGAAGATTGCCCCTCG-3′. WIBR3 hESCs were transfected with pBC2-RFP containing Cas9 and gRNA sequences using Lipo3000. The pBC2-RFP plasmid also contains an RFP gene that can be used to select transfected cells. The cells were then maintained on Matrigel-coated plates. Two days later, RFP-positive cells were sorted via FACS (BD FACSAria Fusion) onto a MEF-coated 6-cm plate for clonal expansion. Knockout of NEAT1 was verified by genomic PCR followed by Sanger sequencing and qRT-PCR.

2.13. RNA sequencing and bioinformatics analysis

RNA-Seq libraries were prepared using VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina (Vzyme) following manufacturer’s instructions. Briefly, total RNA was purified twice using polyT oligo-attached magnetic beads before the synthesis of double-stranded (ds) cDNA. The ds-cDNA was ligated to adaptors (Vzyme) and sequenced by Illumina (Novogene). Fastq data were quality-checked by FastQC (version 0.11.9). 150bp paired-end reads with adapters were trimmed by TrimGalore (v0.6.7) and cutadapt (v4.1). Trimmed reads from each sample were mapped to the human genome (hg38) using STAR (v2.7.10a) with parameters outFilterMismatchNoverLmax = 0.1 and outFilterMultimapNmax = 1. Gene read counts were quantified
against the GENCODE v40 gene annotation using Subread featureCounts (v2.0.1), and then normalized to transcripts per million (TPM) as gene expression level. \( R^2 \) was calculated using python package sklearn (v1.1.1) to reveal reproducibility and difference among samples. Principal component analysis (PCA) was conducted by package sklearn as well. Gene expression data published online with fastq files accessible were downloaded and then analyzed in the same way.

**2.14. Differentially expressed genes (DEGs) and enrichment analysis**

DEGs were identified by DESeq2 (v1.36.0) in R software (v4.2.0), where gene raw counts were inputted. Significance of logarithmic fold changes were determined by a Wald test to approximate \( p \) values. DEGs with thresholds \( p \)-value < 0.05 and \( \log_2 \text{FoldChange} >1 \) or \( \leq -1 \) were labeled as up-regulated and down-regulated, respectively. Up- and down-regulated DEGs overlapped between two knockout clones were then used for gene ontology (GO) enrichment analysis. Gene EnsemblID was converted to EntrzID with R package org.Hs.eg.db (v3.15.0). Gene set enrichment analysis (GSEA) was performed using R package clusterProfiler (v4.4.4) with parameters \( \text{minGSSize} = 100 \), \( \text{maxGSSize} = 1000 \), and EntrzID together with \( \log_2 \text{FoldChange} \) inputting. GO terms with \( p \)-value < 0.05 were defined as significantly enriched.

**2.15. Quantification and statistical analysis**

No statistical methods were used to pre-determine the sample size. No data were excluded from the analyses. Statistical analyses were performed by GraphPad Prism 8, python (v3.9.12), and R software (4.2.0). R package enrichplot (v1.14.0) was used for the generation of GO enrichment network and profile. Python package matplotlib (v3.5.1) and seaborn (v0.11.2) were used for the generation of volcano plot, scatter plot, heatmap, and bar plot. Details of statistics were listed in corresponding figure legends. Significance defined as \( p \)-value was indicated on figures (* \( p \)-value < 0.05, ** \( p \)-value < 0.01, *** \( p \)-value < 0.001, **** \( p \)-value < 0.0001).

**3. Results**

**3.1. Development of a highly uniform and reproducible spheroid assembly strategy**

To generate hESC spheroids, we first tried culturing dissociated WIBR3 hESCs in an ultra-low attachment 96-well round bottom microplate (UL96). Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor Y-27632 was added to prevent the apoptosis of dissociated hESCs [81, 82]. Unfortunately, although the majority of hESCs aggregated into a major spheroid in each well, we observed numerous minor spheroids (~21 ± 7 per well) in all wells (figure 2(A)). To minimize the formation of minor spheroids, we added 0.0125% PF-127 in culture plate, a non-ionic triblock copolymer which is known to reduce non-specific adhesion of cells or proteins [83]. Excitingly, the addition of PF-127 significantly decreased the number of minor spheroids (~13 ± 5 per well) in UL96 microplate. In the meantime, we used non-treated 96-well microplate (NT96) as a control with an expectation that NT96 will give rise to more minor spheroids than UL96. Surprisingly, we did not observe any minor spheroids in NT96 in the presence of PF-127 (figures 2(A) and (B)). The disappearance of minor spheroids was apparently due to the combination of non-treated plate surface and PF-127, since multiple minor spheroids (~9 ± 4 per well) were formed in NT96 without PF-127 treatment (figures 2(A) and (B)). Henceforth, NT96 plus PF-127 treatment is abbreviated as PN96. The major spheroids formed in PN96 displayed extraordinary size uniformity when compared to other three conditions (figure 2(C)). More importantly, we observed high degree of consistency for the size of hESC derived spheroids from experiments performed at different days (figure 2(C)).

Next, we tested different concentration of PF-127 and found that PF-127 at much lower concentration (0.0005%) also worked (figure 2(D)). In addition, 0.0125% PF-127 yielded the smallest variability (\( CV = 2.5\% \)) of spheroid diameter (figure 2(E)). Based on these findings, we selected a concentration of 0.0125% PF-127 to be utilized in our study. Importantly, we found that pre-treatment with 0.0125% PF-127 for 12 h cannot make cells aggregate well in non-treated plastic plate (figures 2(F) and (G)), suggesting a novel mechanism by PF-127 in promoting cell aggregation.

During the process of hESC aggregation after seeding, hESCs have already aggregated into one area in about 1 h after plating in non-treated plate with media containing PF-127. By 3–4 h, the cells had gathered in a tight round area, which eventually transformed into a single spheroid. (figures 2(H) and 2(I)) In contrast, cells in the absence of PF-127 were mostly dispersed, with small clusters forming at various nucleation sites at 1 h, followed by spheroid formation near these clusters (figures 2(H) and S1(A)). These observations suggest that PF-127 starts to function within 1 h of seeding to promote uniform organoid formation.

To investigate the underlying mechanism by which PF-127 leads to uniform organoid formation, we conducted RNA-Seq analyses of hESCs cultured with or without 0.0125% PF-127 at 0, 1, 4, and 24 h. The RNA-Seq analyses revealed that PF-127 had minimal effect on the transcriptome of hESCs in general (figure S1(B)). Interestingly, GO analysis indicated that PF-127 may have significant influence on pathways that regulate protein targeting to the membrane.
Figure 2. PF-127 and Y-27 632 promote the assembly of hESC spheroids. (A) Representative images of hESC aggregation in NT96 or UL96 microplate in the presence (+) or absence (−) of PF-127 24 h after seeding. Scale bars, 200 µm for all images. (B) Representative images of hESC aggregation in one NT96 microplate in the presence of PF-127 24 h after seeding. Scale bars, 500 µm for all images. (C) 3D aggregate diameters of hESC spheroids cultured in NT96 or UL96 microplate in the presence (+) or absence (−) of PF-127. Data represent mean ±SD. Data points from the same experiment are labeled in the same color. n = 36 spheroids for each condition were measured from three independent experiments. (D) Representative images of hESC aggregation treated with different concentrations of PF-127 in NT96 microplate 24 h after seeding. Scale bars, 200 µm for all images. (E) 3D aggregate diameters of hESC spheroids cultured with different concentrations of PF-127 in NT96 microplate 24 h after seeding. Data represent mean ±SD. Data points from the same experiment are labeled in the same color. n = 9 spheroids for each condition were measured from 3 independent experiments. (F) Representative images of hESC aggregation in 0.0125% pretreated microplate or with culture media containing 0.0125% PF-127 in NT96 microplate 24 h after seeding. Scale bars, 200 µm for all images. (G) 3D aggregate diameters of hESC spheroids in 0.0125% pretreated microplate or with culture media containing 0.0125% PF-127 in NT96 microplate 24 h after seeding. Data represent mean ± SD. Data points from the same experiment are labeled in the same color. n = 9 spheroids for each condition were measured from 3 independent experiments. (H) Time-course snapshots of hESC aggregation in NT96 or UL96 microplate in the presence (+) or absence (−) of PF-127 from 40 min to 23 h after seeding. Scale bars, 200 µm for all images. (I) Representative images of hESC aggregation in the presence (+) or absence (−) of PF-127 and Y-27 632 in NT96 microplate 24 h after seeding. Scale bars, 200 µm for all images.

This is very likely relevant to its function in promoting spheroid formation since cell–cell adhesion depends on numerous proteins targeted to cell membrane [84, 85]. To show the generality of PN96 for uniform spheroid formation for other cells, we then tested another hESC line H9 and mouse ESC (mESC) line V6.5. In all cases, relatively uniform spheroids were formed in PN96 (figures S2(A)–(C)). Interestingly, we found that while both ROCK inhibitor Y-27632 and PF-127 were required for the formation of uniform spheroids for hESCs in NT96 (figure 2(I)), only PF-127 alone was required for spheroid formation of mESCs in NT96 (figure S2(D)). Together, these data demonstrate that we have successfully developed a strategy to assemble hESC spheroids with high uniformity.

3.2. Highly uniform and reproducible hepatic organoid formation

In the conventional approach to generate hepatic organoid, a 2D differentiation step and Matrigel embedding are typically required (figure 3(A)) [28, 86–88]. During the 2D differentiation, cell spheroids that form on the 2D cell layer need to be manually handled and embedded into Matrigel using pipettes. This process can be challenging, time-consuming, and may reduce reproducibility. In our method, after spheroids initially formed with PF-127, only
medium changing is performed for culture organoids in a single well all the time (figure 3(B)). Here, we compared liver organoids by our protocol to those formed using Ouchi et al protocol (figures 3(A) and (B)) [86]. We observed that the morphology and size of organoids during the differentiation process by Ouchi et al protocol in our study is consistent with the original paper (figures 3(A) and S3(A)). Luminal structure features typically associated with hepatic organoids were observed using our method (figures 3(B) and S3(B)). Hepatic organoids produced by our method are ten times larger in size (figures 3(C) and S3(A), (B)), which makes it easier for handling for subsequent assays, such as IF or functional assays. Additionally, our method exhibits better uniformity and reproducibility in terms of organoid size and hepatic marker expression (figures 3(C), (D) and S3(B)). Besides, hepatic organoids formed by our method exhibit relatively uniform sizes during the differentiation process and show a high level of batch-to-batch consistency across experiments conducted over a span of more than 3 months (figure S3(C)).

Furthermore, qRT-PCR analysis of hepatic-specific markers, especially mature hepatic markers ((ALB (albumin), A1AT (Alpha 1 Antitrypsin), TF (Transferrin), and TTR (Transthyretin)), indicates that our protocol achieves similar levels of
immunostaining analyses further confirmed the expression of mature hepatic markers (ALB, A1AT, and TF) in hepatic organoids formed by our method (figure 4(A)). Notably, we observed characteristic hepatic-specific functions in hepatic organoids formed by our method. The hepatic organoids consistently exhibited significantly high levels of accumulated glycogen detected through ICG uptake and release and PAS staining (figures 4(B) and (C)). Additionally, lipid storage was detected in hepatic organoids through Oil Red O staining (figure 4(D)).

Furthermore, we observed increased levels of α-fetoprotein, albumin secretion, and urea production activities in hepatic organoids (Albumin, 1.9 ± 0.071 µg ml⁻¹/24 h/10⁶ cells; urea, 137 ± 19 µg ml⁻¹/24 h/10⁶ cells) compared to hESC spheroids (figures 4(E)–(G)), which were comparable to hepatic organoids formed using other protocols at similar time point from previous
studies (Albumin, \(\sim 1.6-3 \mu g \text{ ml}^{-1}/24 \text{ h/10}^5 \text{ cells}\); urea, \(\sim 40-60 \mu g \text{ ml}^{-1}/24 \text{ h/10}^6 \text{ cells}\)) [87, 92]. However, the levels of albumin and urea production were not as high as those found in primary human hepatocytes analyzed by others (Albumin, \(\sim 13 \mu g \text{ ml}^{-1}/24 \text{ h/10}^6 \text{ cells}\); urea, \(\sim 600 \mu g \text{ ml}^{-1}/24 \text{ h/10}^6 \text{ cells}\)) [28].

Together, these results indicate that we have successfully developed a hepatic organoid formation method based on hESC derived spheroids. Moreover, these findings highlight the need for further improvements in inducing mature hepatic phenotypic characters.

3.3. Highly uniform and reproducible nephron, cortical, and lung organoid formation

To show the generality of our methods in organoid culturing, we then performed nephron, cortical, and lung organoid differentiation using our strategy. For nephron organoid formation, we followed a multi-stage differentiation protocol by adding series of growth factors and chemicals essentially as reported previously (figure S4(A)) [93]. qRT-PCR analysis at different timepoints demonstrated successful differentiation towards nephron lineages (figure S4(B)). By day 23, the renal vesicle structures were clearly observed and IF staining showed the formation of loop of Henle/distal tubules (E-cadherin (CDH1\(^+\)), podocytes (PODXL\(^+\), WT1\(^+\)), and nephron progenitor cells (SIX2\(^+\)) (figure S4(C)). Moreover, dextran uptake assay confirmed the renal absorption function of these nephron organoids (figure S4(D)). Finally, size distribution and gene expression analyses demonstrated that our method formed relatively uniform nephron organoids with high batch-to-batch reproducibility (figures S4(E)–(G)).

For cortical organoid formation, we followed a protocol developed by Pasca et al. at 2015 (figure S5(A)) [9]. 3D cortical cortex-like structures with rosette-like neuroepithelia were clearly observed in these cortical organoids after 25 d of differentiation (figure S5(B)). qRT-PCR analysis confirmed successful differentiation towards neuroepithelia and cells of different cortical layers (figure S5(C)). IF staining supported the presence of Cajal–Retzius cells and the formation of TBR1 preplate. Neurons exhibited separation into an early-born deep layer (CTIP2) and a late-born superficial layer (SATB2) (figure S5(D)). Importantly, size distribution and gene expression analyses demonstrated that highly uniform cortical organoids were formed with good batch-to-batch reproducibility (figures S5(E)–(G)). Here, we also compared cortical organoids by our protocol to those formed by spin\(\Omega\) protocol [8]. The variation of size was much smaller for organoids formed by our method (CV = 3.4% at d25-30 formed by our method; 22% formed at d25-30 by spin\(\Omega\) protocol) (figure. S5H). In addition, qRT-PCR analysis of key markers showed more uniform differentiation achieved by ours than spin\(\Omega\) protocol (CV = 3.1% (PAX6), 4.4% (TBR1), 6% (CTIP2), 34% (SATB2), 15% (CUX1), 3.9% (BRN2), 5.5% (RELN) formed by PN96; CV = 6.7% (PAX6), 6.3% (TBR1), 50% (CTIP2), 43% (SATB2), 37% (CUX1), 9% (BRN2), 26% (RELN) formed by spin\(\Omega\) protocol) (figure S5(I)).

Finally, for lung organoid formation (figure S6(A)), we followed a differentiation protocol developed by Spence group [36]. Epithelial structures were clearly observed in differentiated lung organoids (figure S6(A)). qRT-PCR analysis of related markers showed successful lung organoid generation (figure S6(B)). Importantly, size distribution demonstrated that highly uniform lung organoids were formed with good batch-to-batch reproducibility (figures S6(C) and (D)).

3.4. Comparative analysis of organoids generated by our and other strategies through RNA-seq

Next, we performed RNA-Seq for four different type organoids formed by our strategy, including cortical, nephron, hepatic, and lung organoids. PCA indicated that these organoids were clustered correctly and together with the same type of organoids from other studies (figure S7 and tables S1, S2). In addition, cortical organoids generated in this study by our strategy and spin\(\Omega\) protocol were also clustered together (figure S7 and tables S1, S2). These data demonstrate that our methods are robust methods for generating multiple types of organoids from all three germ layers.

3.5. The platform is adaptable to microfluidic chip

Microfluidic devices allow precise control and manipulation of fluid flows in an automated fashion. The application of microfluidic technology in organoid field has shown promises for providing more versatile models for preclinical applications and drug screening [94]. Therefore, we tested whether our platform can be adapted to a microfluidic device. We designed a microfluidic device for automated continuous culturing of organoids in a controlled manner. The device consisted of a two-layer PDMS chip, a fluid control syringe pump, and a disposal bottle (figures S5(A) and S8(A)). The two-layer PDMS chip contained a 16-well chamber and an overlying layer of fluidic channels. The individual chamber units were used for the 3D culture and formation of organoids. The cone-shaped chamber units were 2.5 mm in height, containing 100 \(\mu l\) media (figure S8(B)). The variable fluidic conditions were supplied to the well via channels passing over the top of the chambers. The continuous flow of medium was set at the rate of \(\sim 60 \mu l\) per well per day using a syringe pump. We first tested whether hESCs can form spheroids on the chip in the presence of Y-27632 and PF-127. Consistent with results from PN96 microplate, hESCs formed spheroids with highly uniform size on PDMS chips.
Figure 5. Semi-automated culturing of organoids in microfluidic device. (A) Graphic display for semi-automated organoid culturing on microfluidic chips. In our design, each chip can be simultaneously processed and differentiated without interference to each other. (B) 3D aggregate diameters of hESCs on chips in the presence (+) or absence (−) of PF-127 24 h after seeding. Data represent mean ±SD, n = 8 for each condition. (C) Diameters of hESC spheroids, formed on chips or in NT96 microplate. Data points from the same experiment are labeled in the same color. Data represent mean ±SD, n = 6–16 hepatic organoids were counted for each stage. (D) Relative gene expression levels by qPCR of definitive endoderm, endoderm, and hepatic markers in day 22 hepatic organoids on the chip normalized to hESCs. Data represent mean ±SD, n = 8 for hepatic organoids on chip. (G) Cryosections from the indicated regions of day 17 hepatic organoid on the chip were stained by double immunofluorescence with the indicated antibodies and counterstained with nuclear DAPI. HNF4A (hepatocyte nuclear factor 4α), ALP (α-fetoprotein), and ALB (albumin), hepatic markers. Scale bars, 20 µm. (H) Representative images of day 17 hepatic organoids on the chip in the presence (+) or absence (−) of oxidizing agent after PAS staining. Scale bars, 20 µm. (I) Representative images of day 17 hepatic organoids on the chip in the presence (+) or absence (−) of oxidizing agent after oil red O staining. Scale bars, 20 µm. (J) ELISA measurement of α-fetoprotein in hESC spheroids and day 22 hepatic organoids on the chip. Data represent mean ±SD, n = 4 for hESC spheroids, n = 14 for day 22 hepatic organoids. (K) ELISA measurement of albumin in hESC spheroids. Data represent mean ±SD, n = 3 for hESC spheroids, n = 3 for day 22 hepatic organoids. (L) ELISA measurement of albumin in hepatic organoids on the chip on day 22 and in PN96 on day 19 compared with hESC spheroids. Data represent mean ±SD, n = 13–14 for hepatic organoids, (O) ELISA measurement of albumin in hepatic organoids on the chip on day 22 and in PN96 on day 19 compared with hESC spheroids. Data represent mean ±SD, n = 2–3 for hepatic organoids.

Encouraged by the above results, we further induced hepatic organoid differentiation on PDMS chip based on the similar protocol used for PN96 (figure 5(D)). In the whole process, the media was replaced through the syringe unit, therefore generating minimum interference for organoids. Again, uniform hepatic organoids were generated on PDMS chip (figure 5(E)). qRT-PCR, IF staining, and function analyses showed that hepatic organoids are successfully generated (figures 5(F)–(K)). Furthermore, the hepatic organoids formed by PN96 or chip were comparable in terms of size, gene expression, and
secretion of AFP and ALB (figures 5(L)–(O)). Finally, we also generated cortical organoids using this micro-fluidic chip system (figures S8(D)–(H)). Consistent with the above data, RNA-seq analysis showed that hepatic and cortical organoids formed on chip were clustered together with respective organoids in PN96 (figure S7). Altogether, these results demonstrate that our platform can be adapted to semi-automated microfluidic chip system.

3.6. Tissue specific drug toxicity test in 96-well format
All human tissues could be damaged by toxicity from chemical drugs, with kidney and liver being especially sensitive as they are sites for toxin filtration or metabolism. We reasoned that drug toxicity in different tissues can be comprehensively tested using our method. We first constructed hepatic, nephron, cortical, and lung organoids in PN96. We picked 12 antibiotics from four different classes that are routinely used in clinic to test their toxicity at physiologically relevant concentrations. The viability of organoid cells was measured by quantifying ATP content. Consistent with previous reports [95, 96], Penicillin showed no toxicity, while Ciprofloxacin was highly toxic to organoids of all four tissues (figure 6(A)). Interestingly, aminoglycosides and lactams showed generally low toxicity towards these tissues (figure 6(A)). Roxithromycin is a macrolide antibiotic used to treat infections of a variety of bacteria in tissues such as lung, skin, tonsils, and urinary tract. In our assay, it showed high toxicity towards hepatic and nephron organoids, medium toxicity towards lung organoids, and little toxicity to cortical organoids (figure 6(A)). Dosage response experiments confirmed tissue specific toxicity of Roxithromycin (figure 6(B)). In addition, it was clearly seen that the structures of hepatic and nephron organoids are destroyed by Roxithromycin (figure 6(C)). Together, these data showed the potential of our platform for testing tissue specific drug response in a high throughput fashion.

![Figure 6](image-url)

Figure 6. Tissue specific toxicity test of antibiotics. (A) Application of cortical, nephron, hepatic, and lung organoids for testing viability in response to antibiotics (200 µg ml⁻¹) in PN96. Data represent means ± SD, n = 4–6 from three independent experiments. (B) Cell viability of cortical, nephron, hepatic, and lung organoids 72 h after treatment with various concentrations of roxithromycin, n = 2–6 from 2–3 independent experiments. (C) Representative images of day 14 nephron organoids and day 12 hepatic organoids with or without roxithromycin treatment (200 µg ml⁻¹) for 72 h. Scale bars, 200 µm.
3.7. Identification of tissue specific optimal lipid nanoparticle (LNP) for mRNA transfection

Genetic engineering can provide new insights for gene function or induce functionally important cell types in organoids. However, the optimal transfection reagents for different types of organoids have not been determined, which makes genetic engineering in organoids not feasible. Recent years, LNP has become a useful platform that could deliver nucleic acid into targeted tissue and cells with high efficacy [97]. Therefore, we here want to satisfy organoid transfection via LNP vehicle. Due to very limited knowledge about that, we plan to prepare several LNPs with highly-different physicochemical properties which may help to figure out lead candidate and provide more information on organoids transfection. A selective organ targeting (SORT) strategy have been previously reported which offers a guideline to rationally design LNP [80, 98]. By the inclusion of additional cationic or anionic lipid into conventional four-component LNP, the formed five-component LNP could achieve selective mRNA delivery in specific tissues in vitro [80, 98]. Following this concept, we here performed a proof-of-concept study on whether SORT LNP can be used to optimize mRNA transfection efficiency in different classes of organoids in vitro. Based on SM-102 LNP (marked LNP-2), we prepared two other formulations by adding cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (LNP-1) or anionic lipid 1,2-dioleoyl-sn-glyco-3-phosphate (18PA) (LNP-3). We selected SM-102 LNP as the basal formulation because its safety and efficacy in delivering mRNA have been widely validated. SM-102 LNP was recently approved by FDA for delivering SARS-CoV-2 mRNA vaccine [99, 100]. We then tested transfection efficiency of above three LNPs and commonly used reagent lipofectamine (Lipo3000) via delivering luciferase mRNA into hepatic, nephron, lung, and cortical organoids (figure 7(A)). The results showed that these reagents displayed largely varied efficiencies on different types of organoids. Notably, LNP-3 had highest efficacy in nephron and lung organoids which showed much better efficacy than Lipo3000 (figure 7(A)). While LNP-1 showed the highest signal in cortical and comparable high-signal with Lipo3000 in hepatic organoids. In contrast, LNP-2 had generally low performance even in hepatic organoids (figure 7(A)), indicating that standard formulation is not optimal for organoid transfection even though it works well in vivo. To further test potential organoid-specific delivery, we cultured hepatic, nephron, lung, and cortical organoids in the same well and then performed transfection (figure 7(B)). The results revealed that LNP-3 had selectivity towards nephron organoids, but no obvious organoids tropism was found for other LNPs (figure 7(B)). Together, these results show that SORT technology gives a quick way to figure out optimal LNP vehicle used in organoid transfection. It seems that positive LNP (LNP-1) tends to transfect cortical and hepatic organoids, negative LNP (LNP-3) tends to transfect nephron and lung organoids. These findings may open a window to guide how to optimize mRNA transfection in organoids.

3.8. Functional dissection of NEAT1 IncRNA in human tissue-specific development

Functional divergence for orthologous genes is routinely observed between humans and other model animals [101, 102]. Therefore, organoids representing different human tissues provide unique and necessary models to understand gene function specific for human, especially for genes with less conserved sequences. Long noncoding RNAs (IncRNAs) play vital roles in regulating diverse biological processes and are generally less conserved than protein-coding genes [103, 104]. Their functions in human organ development are generally not well understood. Here we picked IncRNA NEAT1 that have been studied in mouse models [105, 106] and human cells [107] and investigated their functions in human development using our platform. First, we generated NEAT1−/− hESC lines using CRISPR/Cas9 to knock out sequences around its transcriptional start site (figure S9(A)). Successful knockout of NEAT1 was confirmed by genome PCR and RT-qPCR (figures S9(B) and (C)). We noticed that NEAT1 knockout has little impact on the expression of pluripotency genes in hESCs (figure S9(C)). Next, we constructed cortical, nephron, hepatic, and lung organoids from NEAT1−/− and wild type hESCs. We did not observe significant morphological differences or growth phenotype for all four types of organoids derived from NEAT1−/− versus wild type hESCs (figure 8(A)). PCA for global gene expression or lineage markers and correlation analysis showed that all four types of NEAT1 knockout organoids were clustered together with respective wild type organoids (figures 8(B), (C), S10(A) and tables S1, S2), suggesting that NEAT1 knockout has no substantial impact for differentiation of these four lineages. However, a significant number of genes were differentially expressed between wild type and NEAT1 knockout organoids (figure 8(D) and table S1). Moreover, there was little overlap between dysregulated genes by NEAT1 knockout for organoids of different tissues (figures S10(B), (C) and table S1), suggesting that regulation of gene expression by NEAT1 is largely tissue specific.

GO analysis indicated multiple pathways are altered by NEAT1 knockout in hESCs, cortical, nephron, and hepatic but not lung organoids (figures
Figure 7. Tissue-specific transfection efficiency test. (A) Relative luciferase expression in cortical, nephron, hepatic, and lung organoids transfected separately with different transfection reagents. Data were normalized to cortical organoids transfected with Lipo3000. Data represent mean ± SD. n = 6–9 for each condition from 2–3 independent experiments. (B) Relative luciferase expression in cortical, nephron, hepatic, and lung organoids transfected in the same well with different transfection reagents. Data were normalized to cortical organoids transfected with Lipo3000. Data represent mean ± SD. n = 3 for each condition.

S10(D), (E) and tables S3–S8). We found that Wnt signaling pathway is repressed upon NEAT1 knockout in nephron organoids (figure 8(E) and table S3). GSEA independently confirmed that Wnt signaling pathway was inhibited in NEAT1 knockout nephron organoids (figure 8(F) and table S9). Wnt signaling is critical for the induction of nephron epithelial from metanephric mesenchyme (MM) during nephrogenesis [32, 108]. Consistently, MM markers were upregulated and nephron markers (i.e. podocyte markers PODXL and SYNPO) were downregulated in NEAT1 knockout nephron organoids (figure 8(G) and table S1). For hepatic organoids, GO analysis indicated that multiple pathways including Wnt, bone morphogenetic protein (BMP), and MAPK/ERK signaling pathways are altered by NEAT1 knockout (figure 8(E) and table S4). GSEA confirmed that Wnt, BMP, and MAPK/ERK signaling pathways were inhibited in NEAT1 knockout hepatic organoids (figure 8(H) and table S10). Wnt, BMP, and MAPK/ERK signaling are critical for hepatoblast induction and proliferation as well as differentiation of hepatoblast into hepatocytes or cholangiocytes [109, 110]. Consistently, we found that hepatoblast markers were downregulated accompanied with the upregulation of hepatic markers (figure 8(I) and table S1), suggesting that
NEAT1 may finetune the regulation of hepatic differentiation processes. Finally, we found that multiple pathways involved in immune responses are altered by NEAT1 knockout in cortical organoids (figures 8(J), (K) and table S11). Using qRT-PCR, we confirmed that immune related genes are upregulated in NEAT1 knockout cortical organoids (figure 8(L)). Together, these data show that NEAT1 may play important tissue-specific functions in human. In addition, these data support that our platform provides a useful system to make preliminary discovery on dissecting gene function in human development.
4. Discussion

Current organoid culture methods have many drawbacks, including but not limited to the usage of expensive reagents and materials and laborious procedures such as cell re-plating and embedding in extracellular matrices [60]. On top of these limitations, lack of uniformity and reproducibility has decreased the robustness of current organoid models in disease modeling and drug screening [60]. The strategy presented in this study eliminates commonly used hydrogel, AggreWell plate or low attachment plate for the assembly of hESC spheroids and the subsequent differentiation of hESC organoids, therefore significantly lowering the cost of organoid production to 1/20. The use of inexpensive culture plate and chemical PF-127 and simplified procedures render our protocol suitable for most biology laboratories. Moreover, before using AggreWell plates and low attachment plates for organoid construction, the cells need to be centrifuged in order to aggregate. No centrifugation was needed for spheroid formation in our strategy. More importantly, as we showed repeatedly, the protocol generates uniform different kinds of organoids with high reproducibility, paving the way for high throughput perturbation studies such as drug screening and gene function analysis. Finally, our protocol can be adapted to microfluidic devices, showing promises in further reducing the cost and the automation of organoid generation process.

The robustness of our protocol is mainly due to highly uniform spheroid assembly in the presence of PF-127 at the initial step. The concentration of PF-127 used in our strategy to promote the assembly of hESC spheroids is $\sim 10 \ \mu M$, equal to 0.0125% w/v. In fact, even lower concentration (0.0005% w/v) was able to promote spheroid formation. The advantages of using PF-127 include no batch-to-batch variation, easy to operate, determined ingredients, non-toxic, and lower cost. We found that PF-127 may promote spheroid formation in untreated plate through a mechanism distinct from plate coating. Moreover, RNA-Seq analyses at various timepoints indicate that PF-127 may have significant influence on pathways that regulate protein targeting to the membrane. This impact is very likely relevant to its function in promoting spheroid formation since cell–cell adhesion depends on numerous proteins targeted to cell membrane [84, 85]. However, the exact mechanism by PF-127 is still not clear and further investigations are warranted in the future, for example, by proteomics analysis or pathway analysis through genetics or chemical genetics approaches. Dissecting the mechanism behind these phenomena will not only help to develop more advanced methods for spheroid assembly, but also provide mechanistic insights for cell–cell adhesion and self-organization. Recently, spheroids and organoids derived from patient tumor tissues have shown promises as more appropriate pre-clinic models for tumor biology study and cancer drug discovery [111]. Nevertheless, current technologies for generating tumor spheroids and organoids also suffer from challenges such as low uniformity and reproducibility [112]. Therefore, it is important to evaluate the suitability for our platform to generate spheroids and organoids derived from tumor cells in the future. Besides, the ability of PF-127 to promote aggregation of multiple cell types provides a new perspective for co-culture of different cell types. Although a variety of organoids with similar physiological functions in vivo have been constructed, none of the established organoid had the full function of their respective organs due to the lack of mesenchymal cells, vascularization, and microbiota. The introduction of vascular or immune cells into organoids using PF-127 may increase the complexity and maturation of organoids and more realistically mimic in vivo structure and function.

In our study, the successful generation of organoids was supported by characteristic structures, the expression of specific markers, and functional assays. In addition, RNA-seq and PCA analyses demonstrated that organoids were clustered correctly with respective organoids from previous studies. However, to know exact cell lineages of organoids, single cell RNA-seq analyses are needed. Moreover, according to different purposes, the protocol may need to be modified by changing initial number of cells for spheroid assembly, concentration of growth factors or chemicals, and the duration of differentiation. Because the protocol offers a relatively standardized procedure, we expect that organoids of other tissues could also be generated with high uniformity and reproducibility with some modifications. The organoids of different tissues can then serve as blocks for building more sophisticated models to study interactions between different tissues and imitate drug responses of human body in near-physiologic settings.

Most drugs showing good efficacy and safety profiles in preclinical models often fail in subsequent clinical trials, a phenomenon known as ‘valley of death’ [113]. The reason is mainly attributed to species differences in system or organ levels. We envisioned that our organoids culture method could provide a useful uniform platform for evaluating species and tissue specific toxicity of chemical drugs and other type of drugs (e.g. small RNAs or mRNA drugs) at the same time. Adaptation of our platform to microfluidic system will likely further minimizes the cost and efforts for the drug screening. In the future, the microfluidic system can be combined with light-sheet microscopy to achieve real-time dynamic 3D imaging. On the other hand, the chip will be improved to connect different kinds of organoids and thus to study the interactions among different organs. Recently, brain organoids have been used to understand the
function of human specific genes or archaic variants of conserved genes in human evolution [114, 115]. LncRNAs are generally less conserved than protein-coding genes [116]. Dissecting their function in human development and physiology has long been hindered by lack of appropriate in vitro models. Here we demonstrated that our platform can be used to unravel the tissue specific gene regulatory functions of IncRNA NEAT1. We uncovered multiple important signaling pathways including Wnt, BMP, and MAPK that are regulated by NEAT1 in nephron and hepatic organoids. Moreover, we found that knocking out NEAT1 triggered significant immune responses specifically in cortical organoids. How NEAT1 regulate these processes in tissue-specific fashion is currently unknown. A plausible explanation may be attributed to altered retention of specific mRNAs in nuclear paraspeckles [117]. However, extensive investigations in the future are needed to unravel the exact underlying mechanism, which will likely expand our knowledge on IncRNA regulation in human development and diseases. Combined with advances in genome editing technology [118], we anticipate that our platform will provide a powerful model system for understanding species and tissue specific functions of IncRNAs as well as species-specific genetic variants.

5. Conclusion

In conclusion, based on the finding that PF-127 efficiently triggers highly uniform spheroid assembly through a mechanism different from plate coating, we report a relatively standardized strategy for easy to operate, scalable, and reproducible generation of hESC derived organoids at low cost. Using our strategy, organoids of different tissues are successfully generated with high sample-to-sample uniformity and batch-to-batch reproducibility. Furthermore, the platform can be adapted to high-throughput screening and microfluidic device. Finally, we demonstrate that our platform may be used to uncover the tissue-specific gene regulatory function of IncRNAs in human development. Therefore, our study provides a robust platform to advance organoid research and related applications in studying human development and diseases.

Data and materials availability

RNA-seq data in this study have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE240256. RNA-seq data of four organoids from previous studies are available in the NCBI GEO or European Nucleotide Archive (ENA): cortical (GSE80073), nephron (GSE70101), hepatic (GSE128717, GSE180882, GSE115833, and GSE130074), and lung (PRJNA319391). Source data are provided with this paper.

All data that support the findings of this study are included within the article (and any supplementary files).

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Author contributions

X S Z performed all experiments with help from others. G X performed all bioinformatics analyses. H H M and Y Y H helped with the initial identification of PF-127 for spheroid formation and constructed microfluidic device. S J D made NEAT1 knockout hESCs and helped with qRT-PCR analysis. Y X W helped with the construction of RNA-seq libraries. Y F and Q C made LNPs for transfection experiments. All authors were involved in the interpretation of data. Y W conceived and supervised the project. X S Z and Y W wrote the manuscript with help from all other authors.

Conflict of interest

A patent application has been filed relating to the data presented in this study.

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