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Editorial Summary: This protocol describes the long term culture of liver and pancreas 3D organoids from human and mouse, and differentiation of liver organoids *in vitro* and *in vivo*. Methodology for genetic manipulation of these self-renewing organoids is also detailed.

Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation

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ABSTRACT

Adult somatic tissues have proven difficult to expand in vitro, largely due to the complexity of recreating appropriate environmental signals in culture. We have overcome this problem recently and developed culture conditions for adult stem cells that allow the long-term expansion of adult primary tissues from small intestine, stomach, liver and pancreas into self-assembling 3D structures that we have termed organoids. We describe a detailed protocol that describes how to grow adult mouse and human liver and pancreas organoids; from cell isolation and long-term expansion to genetic manipulation in vitro. Liver and pancreas cells grow in a gel-based extracellular matrix (ECM) and a defined medium. The cells can self-organize into organoids that self-renew in vitro whilst retaining their tissue-of-origin commitment, genetic stability and differentiation potential into functional cells in vitro (hepatocytes) and in vivo (hepatocytes and endocrine cells). Genetic modification of these organoids opens up avenues for the manipulation of adult stem cells in vitro, which could facilitate the study of human biology and allow gene correction for regenerative medicine purposes. The complete protocol takes 1-4 weeks to generate self-renewing 3D organoids and perform genetic manipulation experiments and requires basic scientific training to perform.

KEYWORDS: organoid, liver, pancreas, genetic manipulation, stem cell culture

INTRODUCTION

Adult somatic tissue-resident stem cells are gaining much attention for their intrinsic ability to self-renew and differentiate into cell types present in adult tissues, whilst retaining their genetic integrity and commitment to their tissue-of-origin[1]. The recent development of 3D cell culture conditions for adult organs has allowed the in vitro expansion of primary tissues from healthy adult mouse and human tissue as well as from tissue affected by disease (patient-derived tissue). In parallel, the development of human ESC- and iPSC-derived cultures that faithfully recapitulate the differentiation of ectoderm (e.g., retina, brain)[2,3] and endoderm derivatives (stomach, small intestine, liver, lung or thyroid [AU: Please include specific examples])[4,5] has opened up avenues to study human development in vitro in an unprecedented manner.

In our previous studies we developed a 3D culture system that allows the long-term expansion of small intestine[6] and stomach epithelial stem cells in organoid cultures[7]. Here, we describe recently established protocols for the long-term expansion and genetic manipulation of adult liver and pancreas cells in 3D organoid cultures from mouse and human models. We also explain the utility of these organoid cultures in disease modelling[8-13].

Development of the Protocol for Organoid Culture from Liver and Pancreas cells

The liver and pancreas develop from a common population of endodermal progenitors (reviewed in ref 14). Although both organs share several aspects of their morphogenesis and development, they give rise to very distinct functional organs with very different regenerative capacities[14]. The liver is mainly formed of two types of epithelial cells, the hepatocytes and biliary epithelial cells (BECs; also known as cholangiocytes), that, together with several non-epithelial cell types (stellate cells, stromal cells, Kupffer cells and endothelial cells), generate the basic functional structure termed the hepatic lobule[15]. The pancreas is formed of two functionally distinct compartments: the exocrine compartment (comprising of duct and acinar cells) and the endocrine compartment (comprising of the islets of Langerhans). The genes and molecular pathways regulating the embryonic development of these two organs are evolutionarily conserved among all vertebrates studied. These include FGF, HGF, Wnt, BMP, RA and TGFβ pathways that promote progenitor proliferation, migration and survival[14,16-18]. Despite our knowledge of the signalling pathways involved during liver and pancreas development, in vitro expansion of adult pancreas or liver cells has remained a challenge. It was previously shown that primary liver cells could be maintained in culture in the presence of EGF, HGF and the corticoid Dexamethasone[19]. However, these conditions did not allow the long-term expansion of liver progenitors. Similarly, mouse embryonic pancreas cultures able to differentiate into endocrine and exocrine lineages in vitro had been obtained from embryonic progenitor pools[20] or hESCs[21,22]. However, these systems do not allow the long-term expansion of pancreas cells in vitro.

The knowledge acquired from developmental studies, combined with our own expertise in stem cell cultures from adult small intestine[6] and stomach[7], allowed us to define, for the first time, culture conditions for the expansion and differentiation of mouse and human adult primary liver[8,9] and pancreas[10,11] tissue. Thus, by providing an ECM (extracellular matrix) environment together with a combination of growth factors essential during liver development and regeneration (HGF, EGF, FGF, Rspondin-1)[8,15,23], we developed a 3D in vitro culture system for the long-term expansion of adult mouse and human liver cells. Likewise, using similar culture conditions as for the liver organoids, we also established pancreas 3D cultures that allow the long-term expansion of adult mouse and human pancreas ductal cells in vitro. For pancreas organoid cultures, Noggin, but not HGF, is a required growth factor[10].

Organoid cultures for mouse and human liver and pancreas tissue. To develop a long-term expandable adult liver culture we combined Matrigel, which acts as the ECM, together with HGF, EGF, FGF and Rspondin-1. Under these culture conditions both healthy adult mouse liver tissue and damage-induced progenitors can be expanded for months in culture[8] (Fig. 1 & 2). The cellular expansion is exponential, allowing one to obtain up to 10^6 cells from 1 single liver progenitor in ~5-6 weeks. The cells express a mixture of both ductal (*Krt7*, *Krt19, Sox9, Epcam,* MIC1-1C3) (Fig. 3A-B) as well as hepatocyte (*Ttr, HNF4*α) markers[8]. Typically, the expanded cells will self-organize into 3D structures that harbour a $Krt19^+$, single-layered epithelial compartment resembling the hepatic ductal compartment, and a pseudo-stratified compartment that partially resembles the liver bud of an E9-E10 embryo[24] (Fig. 3B).

We have recently adapted this system to the culture of human material. We have reported that single $EpCAM^+$ adult human liver cells expand long-term (>5months) in vitro as 3D organoid cultures[9]. We achieved this by supplementing the medium with a small molecule which raises the levels of cAMP, an activator of ductal proliferation[25], and by blocking TGFβ signalling, which acts to induce differentiation (Table 1). Thus, adult liver tissue from humans, but not mice, requires regulation of TGFβ signalling and cAMP activity for long-term expansion.

In contrast to the remarkable expansion capacity of liver organoid cultures, differentiation into hepatocytes does not occur spontaneously. To differentiate the proliferative cells along the hepatocyte lineage, we defined a differentiation medium. We found that blockade of ductal fate by Notch inhibition, a potent inducer of ductal morphogenesis[26], in combination with the removal of Rspondin-1 and addition of Dexamethasone and BMP facilitated the differentiation of the organoids to a hepatocyte fate, for both mouse and human (Fig. 3A-C)[8,9]. Shifting the cultures to this differentiation medium not only upregulated the expression of classic hepatocyte markers (cytochromes, albumin or Alpha-1-antitrypsin) but endowed the cells with functional hepatocyte characteristics in vitro, such as albumin production, cytochrome activity or bile acid production. Of note, when these hepatocyte-like cells were transplanted into a mouse model of Tyrosinaemia Type I liver disease ($FAH^{-/-}$ mouse model)[8] or into an immunodeficient liver injury model with CCl_4/retrorsine[9], the organoid cells engrafted into the liver tissue, generating clusters of functional hepatocytes in vivo.

The pancreas organoids are entirely composed of ductal (*Sox9*$^{+}$, *Krt19*$^{+}$) cells that express the embryonic progenitor marker *Pdx1*[27]. To expand pancreas ductal cells we used a similar protocol as for liver organoids. Thus, adult pancreas organoid cultures will expand long-term in the presence of EGF, Rspondin-1, FGF10 and Noggin[10]. So far, the direct differentiation of these cells along endocrine lineages in vitro has proven unsuccessful. However, when adult pancreas organoid cells were mixed with embryonic E13 mouse pancreas cells and immediately transplanted into the kidney capsule of immunocompromised mice, the adult-derived organoid cells readily differentiated into fully mature, mono-hormonal (insulin-, glucagon- or somatostatin-positive) cells in vivo[10]. Using similar culture conditions, human pancreas duct cells can be expanded in vitro for 4-5 months[11]. However, their endocrine differentiation potential has not been assessed yet.

Genetic manipulation in organoid cultures. Genetic manipulation has been of crucial importance in understanding the biological function of numerous genes, and has provided knowledge of how mutations in certain genes are the cause of diseases such as cancer and obesity[28-31]. We have recently shown that gene transfer to organoids is feasible and allows the study of gene function as well as gene targeting in vitro in 3D cultures[32]. For example, we successfully investigated the function of RNF43, a novel E3 ubiquitin ligase, by retroviral overexpression in organoid cultures, and so defined it as a negative regulator of the Wnt pathway[33]. Similarly, CRISPR gene targeting allowed us to perform gene correction of the cystic fibrosis transmembrane conductor receptor (CFTR) in intestinal organoids derived from cystic fibrosis patients[13]. These examples demonstrate how retroviral gene overexpression and CRISPR/Cas-mediated gene editing can be used for studying gene function, as well as for the potential application of autologous cell therapy.

Here we describe in detail two methods for gene transfer to liver and pancreas organoid cultures: first, retroviral transduction[12], which allows stable integration of the gene of interest into the genome, and second, liposomal transfection allowing transient transfection[13]. Collectively, these protocols will allow gene function analysis in murine and human organoid cultures by gene overexpression, knockdown or transient transfection. First, organoids are trypsinized to single cells. Following dissociation, retroviral transduction efficiency is further enhanced by the addition of polybrene to the culture media which enhances virus adsorption to target cell membranes[34]. For transfection, genes are delivered using a liposomal agent (Lipofectamine 2000). The single cells are mixed with either virus or liposomal transduction or transfection mix and spin-inoculated. We have found that spin-inoculation is not essential, however it increases the transduction or transfection efficiency significantly. Following spin-inoculation, single cells are incubated in the transduction or transfection mix. Organoids are amenable to transduction by retroviruses, lentiviruses and adenoviruses [12,35,36].

Future applications and potential limitations

Organoid cultures derived from adult liver and pancreas tissues can be very useful for studying human liver and pancreas biology in a primary, non-transformed, physiologically relevant system. The parallel development of genetic engineering tools to manipulate mammalian genomes has opened up the possibility for the genetic modification of stem cells in vitro, which, when combined with 3D culture systems, might facilitate gene correction for regenerative medicine purposes.

As a general tool for the study of all kinds of disease, culturing tissue biopsies from patients enables the study of the molecular mechanisms driving pathologies, whilst at the same time providing a platform for gene editing for true autologous cell therapy. As an example, we have recently demonstrated that liver organoids derived from patients with Alpha-1-Antitrypsin deficiency or Allagile syndrome, who harbour germline mutations in A1AT and Notch pathways, respectively, can partially mimic the phenotype of the patients in vitro[9]. In fact, Alpha-1-Antitrypsin organoids develop A1AT precipitates in the organoid cells, similar to those seen in the liver cells from patients[9]. Additionally, pancreas cancer organoid cultures have been used to model primary cancer tissue in vitro[11].The ease of genetic manipulation using either retroviruses or CRISPR/Cas endonuclease may enable a better understanding of gene function in both pancreas and liver and accelerate the field of personalised medicine for pancreas and liver diseases [Query to AU: Please check, editor has changed text].

A current limitation of this culture system is that it is only suitable for the expansion of the adult epithelial compartment of the tissue. In contrast, human gastric organoids derived from hESC[5] or liver buds derived from hiPSC co-cultured with mesenchymal and endothelial cells[37] have demonstrated the formation of gastric organoids and liver buds containing both mesenchymal and epithelial cells. While these systems do allow the detailed study of tissue development and epithelial-mesenchymal interactions during organogenesis and tissue growth, they do not allow the long-term, exponential expansion of the tissue in vitro.

Overall, long-term, adult stem cell-based organoid cultures represent an emerging field for culturing primary normal and diseased tissue in vitro. Combined with the tools for genome engineering, this 3D organoid culture holds great promise for the study of human liver and pancreas biology as well as the molecular mechanisms underlying disease establishment and progression.

Experimental Design

In this detailed protocol we describe recently established protocols for the long-term expansion and genetic manipulation of adult liver and pancreas organoid cultures. In this section, we discuss the

experimental aspects that need to be considered prior to adapting the protocol for culturing and/or genetically manipulating liver and/or pancreas organoids to each experimentally distinct scenario.

Isolating ductal cells. To isolate duct cells from mouse liver tissue, two alternative procedures can be used depending on the requirement and possibility of perfusing the liver. Perfusing the liver is an option for mouse tissue, specifically when large populations of cells are to be isolated by FACS prior to growing them into organoid cultures. A suitable protocol for liver perfusion is beyond the scope of this procedure, but an example would be ref 38. When liver perfusion cannot be performed, e.g. in the case of small biopsies of human tissue, or when there is no need to isolate large quantities of ductal cells, then the tissue will be processed to enrich for ductal structures. In the latter scenario, it is still possible to further digest ductal structures into a single cell suspension if sorting of cells is needed.

When little starting material is available (e.g. in the case of biopsies) or when the rapid establishment of either mouse or human organoid lines is the primary aim, isolated ductal fragments from both pancreas and liver hand-picked from the bulk preparation (described in detail in Procedures step 1A(ix)) are sufficient to create a robust line in a rapid manner. Although this approach will only enrich ductal cells from the isolation rather than providing a pure population, we find that contaminating (non-ductal) cell types have been outcompeted from the culture following two rounds of passaging. If the purification of ductal cells is required prior to the establishment of the line, then it will be necessary to use FACS. The ductal marker EpCAM could be used to isolate a purely ductal cell population[8,10]. It is important to be aware that growth from single cells is less rapid than from hand-picked ducts.

Establishing organoid lines. The culture of long-term expandable liver and pancreas organoids allows the establishment of organoid lines to investigate aspects of liver and pancreas biology in vitro. Several characteristics can be considered when culturing organoids, such as colony formation efficiency (number of organoids formed), organoid size, cell proliferation rate, organoid morphology and gene expression[8-11]. If human material is to be used, then either genetic manipulation or acquisition of samples from patients of interest will be necessary. When deriving lines from patients, ensure that appropriate ethical approval from relevant institutional and national bodies has been gained. Optimally, tissue pieces of at least $1cm^3$ would be used as the starting material. However, we have succeeded in even isolating organoids from fine needle aspirates. Because of the nature of the material, the time that the biopsy has been outside of the body and its conservation are of critical importance. Tissues should always be transported in cold medium (4°C). Consequently, we have found the variability in isolation efficiency to be greater in human than in mouse organoid lines. However, once cultures are established, the maintenance and expansion potential of the cultures is robust and reproducible.

Differentiation of liver organoids. The differentiation of mouse or human liver organoids into hepatocytes can be achieved in a robust manner using in vitro culture conditions[8,9], which we have provided here as part of the protocol. When cultured using the Expansion medium the cells mainly exhibit a ductal phenotype, and acquisition of hepatocyte fate is seen only in cultures in Differentiation medium. The level of maturity of the differentiated cells can be examined by using gene expression and immunostaining analyses (typical markers: Albumin, HNF4α, ZO-1, Cyp3A). When planning experiments involving the differentiation of liver organoids to hepatocytes, consideration should be given to controls, which are cultured in Expansion medium for the same length of time and from the same starting material. This will identify either the starting material or the differentiation protocol as the area for optimization in the event of problems.

Whilst the differentiation of mouse pancreas organoids into endocrine cell types has been achieved[10], the protocol requires specialized technical knowledge including engraftment into the

kidney capsule of a live animal. The differentiation capacity of human pancreas organoids has not been reported. For these reasons, we describe here only the differentiation of liver organoids and not pancreas organoids.

Histological analysis. For best results, the fixation method will require optimization based on the application, antibody and antigen. The most widely used fixative is paraformaldehyde (4% PFA [wt/vol]), which shows broad specificity. Acetone fixation precipitates large protein molecules and is good for cytological preservation but might result in unwanted side-effects due to cell permeabilisation. We have found that for analyses centered on the detection of antibody signal, whole mount staining is the better method. However, paraffin sectioning followed by histological staining is the preferred method when delineation of cellular morphology is required.

Genetic manipulation. As genetic manipulation of the organoids requires dissociation into single cells, it is highly recommended to optimise the starting density and trypsinization time of the organoids to give optimal cell viability. When genetically manipulating liver or pancreas organoids, a highly recommended aspect to consider prior to the genetic manipulation is to have a transduction or transfection control virus or plasmid that allows quick evaluation of the transduction/transfection efficiency (e.g. a GFP-expressing plasmid). This holds true for the transfection of Platinum-E (Plat-E) cells too, as to ensure a high virus titre the transfection efficiency of Plat-E cells should be equal to or higher than 80%. Additional controls that should be included are mock transduced or transfected organoids to be treated with the selection agent during the selection procedure. The following retroviral vectors from addgene allow transduction efficiency to be evaluated based on the expression of the red fluorescence protein dsRed: pMSCV-loxp-dsRed-loxp- eGFP-Puro-WPRE (32702), pMSCV-loxp-dsRed-loxp- 3xHA-Puro-WPRE (32703) and pMSCV-FLIP-puro-dsRed-GFP-miRNA (32704). Also, the 3xHA epitope can be used to confirm gene overexpression (e.g. by western blot). On the other hand, when performing gene knockdown, prior to organoid transduction, it is advised to validate the corresponding siRNA knockdown efficiency using standard transfection in appropriate cell lines (e.g. HEK293T cells)[12].

MATERIALS

REAGENTS

- Male or female mice [Query to AU: Please can you add the type of mice and age-range of mice you recommend using. Please include supplier details] from any genetic background and aged from 6-weeks to 1.5 years-old can be used **!CAUTION** Experiments that involve rodents must be conducted in compliance with all relevant institutional and governmental regulations. We adhere to UK Home Office legislation and guidelines relating to the Animals (Scientific Procedures) Act 1986.

- Human liver/pancreas samples **!CAUTION** Consult Ethics Review Board regulations and obtain informed consent from all subjects before collecting and processing human tissues. Our approval was granted by a UK National Health Service Research Ethics Committee (NHS REC) under the Human Tissue Act 2004.

- Collagenase D (Roche cat.no. 1108866001)

- Collagenase from *Clostridium histolyticum* (Sigma-Aldrich cat.no. C9407)

- Dispase II (Life Technologies cat.no. 17105-041)

- DNaseI (Sigma-Aldrich cat.no. DN25)
- EBSS (Ca2+/Mg2+) (Thermo Scientific HyClone cat.no. 12349752)
- TrypLE (Life Technologies, cat.no. 12605-028)
- FBS (Life Technologies cat.no.26010066)
- BSA (Sigma-Aldrich cat.no. A8806-1G)
- PBS-1X (Life Technologies cat.no 14190-094)
- DMSO (Sigma-Aldrich cat.no. D8418)

Culture reagents

- BME2 RGF PathClear (Amsbio, cat. no. 3533-005-02)
- Matrigel Matrix, Phenol Red-free (BD cat.no. 356231)
- Advanced DMEM/F12 (Life Technologies cat. no. 12634-010)
- DMEM, high glucose, GlutaMAX, pyruvate (Life Technologies cat. no. 31966-021)
- GlutaMax (100X) (Life Technologies cat. no. 35050-068)
- HEPES 1 M (Life Technologies cat. no. 15630-056)
- Penicillin/Streptomycin (10,000 U/mL)(Life Technologies cat. no. 15140-122)
- N2 supplement 100X (Life Technologies cat. no. 17502-048)
- B27 Supplement 50X, minus vitamin A (Life Technologies cat. no. 12587-010)
- N-Acetylcysteine (Sigma-Aldrich cat.no. A0737-5MG)
- Nicotinamide (Sigma-Aldrich cat.no. N0636)
- Recombinant Human FGF10 (Peprotech cat.no. 100-26)
- Recombinant Human EGF (Peprotech cat.no. AF-100-15)
- Recombinant Human HGF (Peprotech cat. no. 100-39)
- Recombinant Mouse EGF (Life Technologies cat.no. PMG8043)
- [Leu^{15}]-Gastrin I Human (Sigma-Aldrich cat.no. G9145)
- TGF-β inhibitor = A83-01 (TOCRIS cat.no. 2939)
- Forskolin (TOCRIS cat.no. 2939)
- Rho kinase inhibitor Y-27632 dihydrochloride (Sigma-Aldrich cat.no. Y0503)

- Recombinant Human Noggin (Peprotech cat.no. 120-10C)

- Wnt3a conditioned medium: generated from the L-Wnt3a cell line (Clevers lab) in DMEM 10% FBS. Activity of each batch is checked in TOP/FOP assay. Wnt3a production is described in BOX2 **!CAUTION** the cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

- Rspondin 1 (RspoI) conditioned medium: generated from the 293T-HA-RspoI-Fc cell line from Akifumi Ootani (kindly donated by Calvin Kuo, Stanford). The activity of each batch is checked in TOP/FOP assay. RspoI production is described in BOX2 **!CAUTION** the cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

- Recombinant Human FGF-19 (R&D 969-FG-025)

- Recombinant Human BMP7 (Peprotech cat.no. OP1 120-03)

- DAPT (Sigma-Aldrich cat.no. D5942)

- Dexamethasone (Sigma-Aldrich cat.no. D4902)

- PGE-2 (Tocris Bioscience 2296)

- Freezing solution (Life Technologies cat.no 12648-010)

Processing organoids for analysis

- PFA (Sigma-Aldrich, cat.no 158127) **!CAUTION** contains formaldehyde, which can cause cancer; handle using appropriate safety gear.

- Acetone (Sigma-Aldrich, cat.no. 34850) **!CAUTION** Flammable. Vapors may cause irritation; handle with care.

- Xylene (Sigma-Aldrich, cat.no. 534056) **!CAUTION** Flammable. Vapors may cause irritation; handle with care.

- n-Butanol (Sigma-Aldrich, cat.no. B7906) **!CAUTION** Flammable. Vapors may cause irritation; handle with care.

- Ethanol 100% and 96% (Sigma-Aldrich, cat.no. 51976)

- Direct-PCR solution (Viagen, cat.no. 102-T)

- Proteinase K (NEB cat.no. P8107S)

- DNeasy Blood & Tissue kit (Qiagen, cat.no. 69504)

- RNeasy Mini kit (Qiagen, cat.no. 74104)

- Normal donkey serum (Sigma-Aldrich, cat.no. D9663-10ml)

- Universal blocking solution (BioGenex, cat.no. HK085-5KE)

- Hoechst 33342(Life Technologies cat.no. H3570)
- Vectashield mounting media with DAPI (Vector, cat.no.H1500)
- Vectashield mounting media without DAPI (Vector, cat.no.H1000)
- Eosin aqueous 1% (Thermo Scientific cat.no. LAMB-100-D)
- Histowax (Thermofisher, cat.no. 8331)
- Antibodies

Transfection and Transduction

- Platinum-E (Plat-E) cells (Cellbiolabs, cat.no. RV-102). **!CAUTION** the cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- Lipofectamine 2000 (Life Technologies, cat.no. 11668-019)
- Opti-MEM Reduced serum media, Glutamax (Life Technologies, cat.no. 51985-026)
- Polybrene (Sigma-Aldrich, cat.no. 107689-10G)

EQUIPMENT

Common equipment

- Biological safety cabinet (e.g. Thermo Scientific, cat.no. 1316)
- Safety Bunsen Burner for safety cabinets (optional)
- Cell culture incubator with 5% CO_2, 37°C (e.g. Panasonic, cat.no. MCO-18AC-PE)
- Brightfield microscope (e.g. Biocompare, cat.no. TS100/TS100F)
- High speed centrifuge (e.g. Eppendorf, cat.no. 5810 R)
- Water Bath 37°C (Thermo Scientific, cat.no. BLE-300-010T)
- Temperature-controlled shaker set to 37 °C (e.g. LT-X (*Lab*-Therm), cat. no. SMX1700)
- Fine scissors (Fine Science Tools, cat.no. 91460-11)
- 2 Fine forceps (Fisher, cat.no. DKC-770-R)
- Pipet-aid, serological pipettes (10ml, VWR, cat.no. 734-1738) and glass Pasteur pipette (150mm length, unplugged, Fisher Scientific, cat.no. 10006021)
- Calibrated pipettes (P10, P1000, P200, P20, P2, e.g. Sigma, Z740362) and sterilized pipette tips. When working with viruses, use filter tips (Starlab, 0.1-10µl filter tips cat.no. S1120-3810, 20µl filter tips cat.no. S1120-1810, 100-200µl filter tips cat.no. S1120-8810, Racked, 100-1000µl filter tips cat.no. S1122-1830).

- Conical centrifuge tubes (SLS, 15 mL cat.no. 352096 and 50mL, cat.no. 352070)
- Microcentrifuge tubes (1.5 mL, Fisher Scientific, cat.no. 30125150)
- FACS tubes (or other tubes suitable for the cell sorter) (e.g. BD falcon, cat.no. 352063)
- Cell strainer 70µm (Falcon, cat.no. 352350)
- Cell strainer 40µm (Falcon, cat.no. 352340)
- 100mm Petri dish (VWR, cat.no. 470151-288)
- 48 well Suspension culture plate (GreinerBio-one cat.no.677 102)
- 24 well Suspension culture plate (GreinerBio-one cat.no.662 102)
- 24 well Adherent culture plate (Thermo Scientific Nunclon cat.no. 142475)
- Cell freezing container CoolCell LX (Biocision cat.no. BCS-405)
- Cryogenic vials 2mL (VWR, cat.no. 479-0287)
- Standard haemocytometer (ImmuneSystems, cat.no. BVS100 or Sigma-Aldrich, cat.no. BR719520)
- Dry ice
- Ice

Processing organoids for analysis

- Plastic Pasteur pipettes 7ml (VWR, cat.no. 612-1681)
- 5ml flat-bottom glass tubes (Fisher Scientific cat.no.11563542)
- Incubator pre-warmed to 65°C (Memmert Incubator, cat.no. LAB129)
- Tissue cassette (VWR, cat.no. 720-0228)
- Glass slides (VWR, 631-9483)
- Coverslips (Fisher Scientific, 15356429)
- Paraffin embedding equipment (Leica, cat.no. EG1150C)

Transfection and Transduction

- Parafilm (Parafilm cat.no. PM-996)
- High speed centrifuge pre-warmed to 32°C (e.g. Eppendorf, 5810R)

REAGENT SETUP

Human liver and/or pancreas samples can be obtained by a trained surgeon as biopsies from the liver or pancreas of a donor during transplantation. At least 1cm^3 of tissue should be collected and processed as soon as possible. After surgical excision the tissue is kept cold at 4 °C in Advanced/DMEMF12 or Wisconsin Solution until processing. **ΔCRITICAL** Although we have successfully isolated material from up to 4 days after isolation, a delay of more than 2 days results in a significant drop in organoid formation efficiency and should be avoided.

Digestion solution should be prepared fresh and used immediately. Dissolve Collagenase and Dispase II in sterile Wash medium (see below) to a concentration of 0.125mg/ml each. Supplement with 0.1mg/ml of DNaseI (dissolved in sterile H_2O). **ΔCRITICAL** Care should be taken to have the correct amounts of Collagenase and Dispase in the Digestion solution, as inaccuracies will have a large impact on the success of the duct isolation.

Human liver digestion solution should be prepared fresh and used immediately. Dissolve lyophilized Collagenase D in sterile EBSS medium to a final concentration of 2.5mg/ml (Collagenase D). Supplement with 0.1mg/ml DNaseI. **ΔCRITICAL** Care should be taken to have the correct amounts of Collagenase and Dispase in the Digestion solution, as inaccuracies will have a large impact on the success of the duct isolation.

PBSB PBS-1X supplemented with 0.1% BSA *(used to dilute all the growth factors)*. Store at 4°C for up to 1 month.

PBSDT blocking solution PBS-1X supplemented with 0.1% - 1% Triton X-100 (depending on the protein localisation membrane/nucleus), 1% DMSO, 1% BSA and 1% serum (from the animal in which the secondary antibodies were raised). Stored at 4°C for up to one week.

Basal Medium: Advanced DMEM/F12 supplemented with 1% Penicillin/Streptomycin, 1% Glutamax, and HEPES 10 mM. Store at 4 °C for up to one month. **ΔCRITICAL** *see supplementary figure 1 for a comparative summary of the composition of all the complete media used in the procedure.*

Wash medium: DMEM (high glucose, GlutaMAX, pyruvate) supplemented with 1% FBS and 1% Penicillin/Streptomycin. Store at 4 °C for up to one month.

Mouse liver Expansion medium: Basal medium supplemented with 1:50 B27 supplement (with or without Vitamin A), 1mM n-Acetylcysteine, 5% (vol/vol) Rspondin-1 conditioned medium, 10mM nicotinamide, 10nM recombinant human [Leu^{15}]-Gastrin I, 50ng/ml recombinant mouse EGF, 100ng/ml recombinant human FGF10, and 50ng/ml recombinant human HGF. Store at 4 °C for up to two weeks.

Human liver Expansion medium: Basal medium supplemented with 1:50 B27 supplement (without Vitamin A), 1:100 N2 supplement, 1mM n-Acetylcysteine, 10% (vol/vol) Rspondin-1 conditioned medium, 10mM nicotinamide, 10nM recombinant human [Leu^{15}]-Gastrin I, 50ng/ml recombinant human EGF, 100ng/ml recombinant human FGF10, 25ng/ml recombinant human HGF, 10µM Forskolin and 5µM A83-01. Store at 4 °C for up to two weeks.

Mouse liver Isolation medium: mouse liver Expansion medium supplemented with 25ng/ml recombinant human Noggin or 5% (vol/vol) Noggin conditioned medium, 30% (vol/vol) Wnt3a conditioned medium and 10µM ROCK inhibitor [Y-27632] (if single cells). Store at 4 °C for up to two weeks.

Human liver Isolation medium: *Human liver Expansion medium* supplemented with 25ng/ml recombinant human Noggin or 5% (vol/vol) Noggin conditioned medium, 30% (vol/vol) Wnt3a conditioned medium and 10µM ROCK inhibitor [Y-27632]. Store at 4 °C for up to two weeks.

Mouse pancreas Expansion and Isolation medium: Basal medium supplemented with 1:50 B27 supplement (without Vitamin A), 1mM n-Acetylcysteine, 5% (vol/vol) Rspondin-1 conditioned medium, 10mM Nicotinamide, 10nM recombinant human [Leu^{15}]-Gastrin I, 50ng/ml recombinant mouse EGF, 100ng/ml recombinant human FGF10 and 25ng/ml recombinant human Noggin or 5% (vol/vol) Noggin conditioned medium. Store at 4 °C for up to two weeks.

Human pancreas Expansion and Isolation medium: Basal medium supplemented with 1:50 B27 supplement (without Vitamin A), 1:100 N2 supplement (optional), 1mM n-Acetylcysteine, 30% (vol/vol) Wnt3a conditioned medium, 5% (vol/vol) Rspondin-1 conditioned medium, 10mM nicotinamide, 10nM recombinant human [Leu^{15}]-Gastrin I, 50ng/ml recombinant human EGF, 100ng/ml recombinant human FGF10, 25ng/ml recombinant human Noggin or 5% (vol/vol) Noggin conditioned medium, 5µM A83-01 and 3µM PGE-2. Store at 4 °C for up to two weeks.

Mouse liver Differentiation medium: Basal medium supplemented with 1:50 B27 supplement (with or without Vitamin A), 1mM n-Acetylcysteine, 10nM recombinant human [Leu^{15}]-Gastrin I, 50ng/ml recombinant mouse EGF, 100ng/ml recombinant human FGF10, 50 nM A83-01 and 10µM DAPT. Supplement Differentiation medium with 3µM Dexamethasone from day 13 to day 15. Store at 4 °C for up to two weeks.

Human liver Differentiation medium: Basal medium supplemented with 1:50 B27 supplement (with Vitamin A), 1:100 N2 supplement, 1mM n-Acetylcysteine, 10nM recombinant human [Leu^{15}]-Gastrin I, 50ng/ml recombinant human EGF, 25ng/ml recombinant human HGF, 0.5µM A83-01, 10µM DAPT, 3µM Dexamethasone, 25ng/ml BMP7 and 100ng/ml recombinant human FGF19. Store at 4 °C for up to two weeks.

Mouse liver Transduction medium: *Mouse liver Isolation medium* supplemented with 8µg/ml *polybrene* and 10µM ROCK inhibitor [Y-27632]. Store at 4 °C for up to two weeks.

Human liver Transduction medium: *Human liver isolation medium* supplemented with 8µg/ml *polybrene*. Store at 4 °C for up to two weeks.

PROCEDURE

Culture of mouse and human liver and pancreas organoids: Isolation of duct cells ●

TIMING 4h

1| Choose from the following options depending on the tissue to process. Mouse liver, mouse pancreas and human pancreas are processed using option A; for human liver follow option B.

(A) Mouse liver, mouse pancreas and human pancreas dissociation

(i) *Tissue collection.* For mouse tissue collection, euthanize the mice using an appropriate ethically approved method, and then remove the liver or pancreas as entire organs by standard surgical procedures. For human tissue collection, after surgical excision keep the tissue cold at 4°C in Basal media until ready for processing**. ΔCRITICAL STEP** The common methods of mouse euthanasia do not adversely affect organoid isolation or culture.

■**PAUSE POINT** Mouse and human tissue can be stored in Basal medium at 4°C for up to 48h.

(ii) Transfer the tissue to the biological safety cabinet and place it into a 100mm Petri dish. Pre-warm Digestion solution to 37°C.

(iii) Mince tissue into pieces of roughly 0.5mm^3 using fine scissors. Transfer minced tissue to a 15ml centrifuge tube and add ~10ml of ice cold Wash medium. Pipette up and down with a 10ml pipette to remove red blood cells and fat (which will float to the top of the solution).

(iv) Allow tissue pieces to settle and discard ~7.5ml of the supernatant, including any floating pieces of fat. Repeat this wash step once.

(v) Remove any remaining wash medium and add ~10ml of the Digestion solution pre-warmed to 37°C. Incubate digestion mixture on a shaker at 37°C for 45min. **ΔCRITICAL STEP** Care should be taken to have the correct amounts of Collagenase and Dispase in the Digestion solution, as inaccuracies will have a large impact on the success of the duct isolation.

(vi) Pipette Digestion solution up and down with a 10ml pipette and check an aliquot of the solution for the presence of clean duct structures using a brightfield microscope (for examples see Fig. 2A and B, day 0 Duct isolation panels). If none are present, return the solution to the shaker at 37°C. Perform this check every 20-30min. **ΔCRITICAL STEP** Generally a large proportion of ducts will appear once no more tissue remains to the naked eye, which will require ~2h total incubation.

(vii) When the ductal structures appear, transfer the supernatant to a fresh 15ml centrifuge tube, add ice cold Wash medium to increase the volume to 15ml and pellet the material at 100-300*g* for 5min at 8°C. Discard supernatant and add cold Wash medium to a volume of 15ml, repeat the pelleting procedure again to wash out any remaining Digestion solution.

(viii) If you wish to cell sort to enrich for ductal cells, follow Box 1 instructions. If sorting is not required, proceed to next step.

(ix) *Enrichment of duct stem cells by hand-picking.* Under aseptic conditions, resuspend the material from step *1(A)*(viii) in 5ml of cold Wash medium and transfer it to a sterile

100mm petri dish. Using a brightfield microscope, identify and collect ducts using a 200µl pipette, transfer material to a 15ml centrifuge tube. **ΔCRITICAL STEP** The goal is not to achieve a pure duct culture but rather to enrich the ducts from any contaminating structures/cells.

? TROUBLESHOOTING

(x) Following the picking of all ducts, add 10ml of Basal Medium to the 15ml centrifuge tube and pellet the material by centrifuging at 100-200*g* for 5min at 8°C. Remove and discard the supernatant before washing the pellet again with 10ml of Basal medium, again pelleting the material by centrifuging at 100-200*g* for 5min at 8°C. The pellet now contains isolated duct fragments that can be directly cultured (see Fig. 2 for example).

(xi) Proceed to step 2.

(B) Human liver dissociation ● TIMING 2h

(i) After surgical excision the tissue is kept cold at 4°C in Basal media until processing.
■PAUSE POINT Tissue can be stored in Basal medium at 4°C for up to 48h.

(ii) Under aseptic conditions, place tissue into a 100mm Petri dish and mince into pieces of roughly 0.5-1mm^3 using fine scissors.

(iii) Transfer minced tissue into a 15ml centrifuge tube and add ~10ml of ice cold Wash medium. Gently pipette up and down with a 10ml pipette to wash the liver pieces. Allow tissue pieces to settle and discard ~7.5ml of the supernatant, including any blood cells and floating pieces of fat. Repeat this wash step once.

(iv) Remove as much supernatant as possible from the pellet, add ~4-5ml per gram of tissue of pre-warmed human liver Digestion solution and incubate at 37°C. **ΔCRITICAL STEP** Care should be taken to have the correct amount of Collagenase in the Digestion solution, as inaccuracies will have a large impact on the success of the dissociation.

(v) After 30min of incubation, pipette the digestion solution up and down vigorously with a 10ml pipette and check an aliquot of the solution for the presence of single cells. If few single cells are present then return the solution to 37°C. Perform this check every 10min for up to a maximum of 90min. Stop the digestion when the suspension contains 80%-100% single cells. **ΔCRITICAL STEP** Take great care not to over-digest the material.

(vi) When digestion is complete, add cold Wash medium to increase the volume to 15ml. Filter the digested material through a 70µm filter, and add ice cold Wash medium to increase the volume to 50ml.

(vii) Pellet the material by centrifuging at 300g for 5min at 8°C. Discard the supernatant and add cold Wash medium to a volume of 15ml. Transfer the resuspended material into a 15ml centrifuge tube.

(viii) Pellet the material by centrifuging at 300g for 5min at 8°C. Remove and discard the supernatant and wash pellet again twice with Wash medium and once with 10ml of Basal medium for the final wash, each time pelleting the material by centrifuging at 300g for 5min at 8°C.

? TROUBLESHOOTING

(ix) If you wish to enrich for ductal cells prior to growth of organoids, sort cells as described in Box 1 prior to proceeding to step 2. If cell sorting is not required, proceed to step 2.

Seeding of duct cells ● TIMING 30 min

ΔCRITICAL Pre-warm tissue culture plates at 37°C for 1h-overnight. For mouse cells, adherent plates can be used. For human cells, suspension plates are required to avoid cell attachment to the plate.

2| Remove the supernatant and resuspend the desired number of cells or duct structures (1000 cells or 50 duct structures per well of a 24-well plate) in an appropriate basement matrix for seeding (i.e. Matrigel for mouse material; BME2 for human material). Use a volume of 50µl/24-well and 25µl/48-well. ΔCRITICAL STEP Basement Matrix (BME2 and Matrigel) require thawing on ice at 4°C overnight. Basement matrix will solidify at room temperature (25°C), so it is important to work quickly and keep the basement matrix cold throughout the process.

3| Seed the material by adding a droplet of basement matrix to the centre of each well, preventing the drop from touching the edges as this will cause the cells to attach to the plate. Incubate 5-10min at 37°C or until the basement matrix is solidified.

4| Overlay the droplet with Isolation medium (500µl/well for a 24-well plate and 250µl/well for a 48-well plate) according to the corresponding tissue as specified in the reagent set up and supplementary Table 1 [Query to AU: Please check, editor has changed text].

5| Incubate material under standard tissue culture conditions (37°C, 5% CO_2).

6| In the case of mouse and human liver, after 3-4 days replace the Isolation medium with normal liver Expansion medium (without Wnt and Noggin). If seeding single cells, then continue to supplement the medium with 10µM of ROCK inhibitor [Y-27632] for the first 6 days in culture. Change medium every 3-4 days. Organoids should be visible within 7 days and ready for passaging before 14 days of culture.

? TROUBLESHOOTING

Cell culture: Passaging organoids for maintenance ● TIMING 30-40min

ΔCRITICAL Pre-warm multi-well plates for seeding at 37°C 1h-overnight.

7| Grow the organoids for 10-15 days after isolation or 5-7 days between passages. ΔCRITICAL STEP The interval between passages may differ slightly depending on the initial density of organoids seeded after isolation. Prevent organoids from overgrowing (i.e. the medium turning yellow). Similarly, do not passage the organoids too early. The general split ratio for a confluent well is 1:4 -1:6.

8| Disrupt the basement matrix containing the organoids (drops in centre of the well) by scraping and pipetting up and down using 500-1000ul of basal medium and a 1000µl pipette. Transfer the organoid suspension to a 15ml centrifuge tube, add cold Basal medium to the top (~13-14ml) and pipette up and down 3-5 times to remove the basement matrix [Query to AU: Please add the volume of Basal medium used]. ΔCRITICAL STEP Do not pool more than 3 wells (24-well plates) or 6 wells (48-well plates) together so the basement matrix can be properly washed from the organoids.

9| Centrifuge the tube at 100-200g for 5min at 8°C. Aspirate the supernatant, leaving ~2ml of it in the tube. Resuspend the organoids in this remaining medium.

10| Flame a glass Pasteur pipette to narrow the aperture (by ~1/2). Using this narrowed Pasteur pipette, pipette up/down 5-10 times the 2ml of supernatant remaining in order to break the organoids. **ΔCRITICAL STEP** Do not dissociate the organoids into single cells (Fig. 2). If a Pasteur pipette cannot be narrowed, use 200µl filter tips and pipette up and down until organoids are completely disrupted into fragments.

11| Add cold Basal medium to fill the tube and centrifuge the tube at 200-250g for 5min at 8°C.

12| Aspirate the supernatant completely. At this step it is possible to freeze the organoids for storage. If freezing organoids follow the procedure described in Box 3.
? TROUBLESHOOTING

13| Suspend the cell aggregates in the appropriate basement matrix (50 µl per well [24-well plates] or 25 µl per well [48-well plates]). Gently pipette up/down until the cell aggregates are completely resuspended. Then seed 50 µl or 25µl basement matrix suspension as a droplet into the centre of each required well on a pre-warmed 24/48-well plate. **ΔCRITICAL STEP** Work quickly and keep the suspension cold to prevent the matrix from solidifying in the tube. Avoid generating air bubbles.
? TROUBLESHOOTING

14| Incubate the plate for 5-10min at 37°C or until the basement matrix polymerises.

15| Overlay with the appropriate Expansion medium in each well (500 µl per well [24-well plates] or 250 µl per well [48-well plates]).

16| Change the medium every 3-4 days and passage organoids by repeating steps 8-15 every 5-7 days as desired. If you wish to differentiate the cells within liver organoids to hepatocytes, follow the procedure described in Box 4. If you wish to perform genetic manipulation on the organoids, proceed to step 17. If you wish to analyse organoids without performing genetic manipulation skip ahead to step 18.
? TROUBLESHOOTING

Genetic manipulation of organoids

17| Perform genetic manipulation of organoids via transfection (option A) or transduction (option B)

(A) Transfection of organoids ● TIMING 1.5h preparation of single cells; 20 min preparation of Lipofectamine and DNA; transfection 3-5h; seeding 20-30 min; selection 2-3h by FACS or 1-2 weeks by antibiotic selection.

(i) *Preparation of single cell mixture.* Culture the organoids as described in steps 7-16 for 5-7 days until 80-90% confluent and then disrupt the basement matrix containing the organoids (drops in centre of the well) by scraping and pipetting up and down using a 1000µl pipette. Pool 3 wells of a 24-well plate or 6 wells of a 48-well plate of organoids in a 15ml centrifuge tube. Add ice-cold Basal media to the top and pipette up and down 3-5 times to remove the basement matrix. Centrifuge the tube at 200g for 5min at room temperature. **ΔCRITICAL STEP** Generally, organoids are ready for transfection 3-4 days after high-density seeding.

(ii) Prepare 2 narrowed glass Pasteur pipettes per transfection condition by flaming a glass Pasteur pipette to make the aperture thinner (reduced to ~1/4 of original aperture size).

(iii) Remove the supernatant from the pellet generated above using a 1000µl pipette to remove the last ml of medium. Try to remove as much supernatant as possible without disturbing the pellet.

(iv) Resuspend the organoids in 2ml of pre-warmed TrypLE Express per tube. Vigorously pipette up and down ~10 times using the narrowed glass Pasteur pipette and incubate at 37°C for 5min. Check the TrypLE digest solution every 2min using a brightfield microscope. Stop the digestion when the majority (90-95%) of material consists of single cells. **ΔCRITICAL STEP** Do not allow the organoid fragments to dissociate for too long, as this results in decreased viability.

(v) Add 10ml of cold Basal medium to stop the digestion and centrifuge the tube at 300-400*g* for 5min at 4°C.

(vi) Remove the supernatant and resuspend the pellet in 450µl of the appropriate medium (i.e. human liver Isolation medium; mouse liver Isolation medium). Keep on ice until transfection.

(vii) *Preparation of Lipofectamine and DNA*. Add 50µl of Opti-MEM per tube to two 1.5ml microcentrifuge tubes for each transfection condition. Add 1µl of DNA at 0.8µg/µl to one of the tubes and 4µl of Lipofectamine 2000 reagent to the other tube.

(viii) Incubate both tubes at room temperature for 5min.

(ix) Mix the content of the two tubes together and incubate at room temperature for a further 5-15min.

(x) *Transfection.* Add 50µl of the DNA:Lipofectamine mixture to 450µl of single cell suspension and transfer this new mixture to one well of a 24-well plate.

(xi) Seal the plate with parafilm and centrifuge in a pre-warmed centrifuge at 32°C, 600*g* for 1h.

(xii) Remove the parafilm and incubate in a tissue culture incubator at 37°C for 2-4h.

(xiii) *Seeding and Selection.* Transfer the transfection mix from the well to a microcentrifuge tube.

(xiv) Centrifuge at 400*g* for 5min at room temperature.

(xv) Remove the medium and proceed according to steps 13 to 14. Overlay with the appropriate Isolation medium.

? TROUBLESHOOTING

(xvi) Select transfected cells from 2-3 days after transfection via FACS enrichment or antibiotic selection. To use the FACS-enrichment approach, prepare a single cell suspension as described above (see steps *17(A)*(i)-(v)), sort (e.g. GFP-expressing plasmid, see BOX1 *steps 5 to 11*) and seed 50 cells/well of a 48-well plate (as described in steps 13 to 14) into the appropriate Isolation medium. To use antibiotic selection, add antibiotic to the appropriate Expansion medium and replace this medium every 2-3 days, supplementing medium with 10µM of ROCK inhibitor [Y-27632] during the selection step. If using antibiotic selection, surviving, stable clones should appear in 5-8 days. If transfecting liver organoids, you may proceed to Box 4 if you wish to differentiate the cells to hepatocytes.

? TROUBLESHOOTING

(B) Viral transduction of organoids ● TIMING 5-6d (~1h hands-on time) for transfection of Plat-E cells; 12.5-16.5h for virus collection; 1h for preparation of viral solution and cell suspension; 7-8h (~1h hands-on time) for retroviral transduction; and ~1-2 weeks for selection

(i) *Transfection of Platinum-E (Plat-E) cells for Virus production.* Day0: Seed approximately $5x10^6$ cells into a 150mm dish with 20ml of medium (DMEM + 10% FBS) in the presence of Puromycin (1µg/ml) and Blasticidin (10µg/ml).

(ii) Day2-3: After 2-3 days, when the Plat-E cells have reached 30-40% confluence, change the medium to 15ml of DMEM + 10% FBS without Puromycin and Blasticidin prior to transfection.

(iii) Prepare two tubes containing 12µg of retroviral DNA construct in 750µl of Opti-MEM. Prepare one tube containing 60µl Lipofectamine 2000 in 1.5ml Opti-MEM. Incubate both tubes at room temperature for 5min.

(iv) Add the 750µl of the Lipofectamine:Opti-MEM solution mix to the 750µl of DNA:Opti-MEM solution. Mix by pipetting and incubate at room temperature for 25-30min.

(v) Add the complete mixture to the medium of Plat-E cells and carefully shake the plate to ensure equal distribution of the DNA:Lipofectamine complexes.

(vi) Incubate the Plat-E cells with the transfection mixture overnight.

(vii) Day3-4: The following day change the medium (DMEM + 10% FBS without Puromycin and Blasticidin) to remove the transfection reagents.

(viii) Culture the cells in this medium for 2 days.

(ix) *Virus collection.* Day5-6: Attach a 0.45µm filter to a 25ml syringe and place it on top of a 50ml ultracentrifuge tube. Remove the piston and add all media from the 150mm dish using a 25ml pipette and pipette boy. Add the piston and pass the media through the filter. **!CAUTION** Be careful that the ultracentrifuge tube does not fall over. If unsure use a retort stand to hold the tube.

(x) Spin the tube at 4°C, 8000*g* for 12–16h.

(xi) *Preparation of the viral solution and cell suspension.* Day6-7: Carefully collect the tube from the ultracentrifuge. A small white pellet should be visible.

? TROUBLESHOOTING

(xii) Transfer the pellet in 500µl – 1ml of the culture medium from the high speed centrifuge tube to a 1.5ml microcentrifuge tube using a 1000µl pipette.

(xiii) Spin the tube at room temperature, 8000*g* for 5min.

(xiv) Discard the supernatant and resuspend the pellet in 250µl of transduction medium. **ΔCRITICAL STEP** To avoid losing the pellet do not aspirate off the supernatant. Instead use a 1000µl pipette followed by a 200µl pipette to remove the supernatant. Try to remove as much supernatant as possible without disturbing the pellet.

(xv) *Preparation of organoid cells for transduction.* Dissociate the organoids to single cells as described in steps *17(A)* (i)-(v).

(xvi) Remove the supernatant, resuspend the cells in 1ml of complete medium and transfer the cells to a 1.5ml tube.

(xvii) Centrifuge at room temperature, 600*g* for 5min.

(xviii) Remove the supernatant and add 20µl of Transduction medium containing polybrene. Resuspend the pellet by tapping the tube.

(xix) Keep on ice at 4°C.

(xx) *Retroviral transduction.* Day6-7: Combine organoid fragments with the 250µl of the retroviral solution from steps *17(B)* (xiv) into one well of a 48-well plate. Mix gently by slowly pipetting using a 1000µl pipette. Seal the plate with parafilm.

(xxi) Centrifuge the plate at 32°C, 600*g* for 1h.

(xxii) Carefully remove the parafilm and incubate the plate at 37°C for 5-6h.

(xxiii) Transfer the infected organoid fragments and Transduction medium from the well to a 1.5ml tube and spin at room temperature, 600*g* for 5min.

(xxiv) Discard the supernatant and incubate the tube containing the pellet on ice for 5min to cool it down. Add 25µl of the appropriate basement matrix and resuspend the pellet by pipetting slowly up and down.

(xxv) Seed the material by adding a 25µl droplet of basement matrix:cell mix to the centre of each well [48-well plate]. Incubate 5-10min at 37°C or until the basement matrix is solidified. Add 250µl Transduction medium without polybrene to the wells and incubate the plate in a tissue culture incubator under standard culture conditions (37°C, 5% CO_2).
? TROUBLESHOOTING

(xxvi) *Selection.* Start selection after 2-3 days by adding puromycin (1µg/ml) to the Expansion medium appropriate for the organoid type being cultured. For mouse organoids, if possible, use medium supplemented with Wnt3a and 10µM of ROCK inhibitor [Y-27632]. **ΔCRITICAL STEP** It is important not to wait longer than 2-3 days to start selection. Non-infected organoids will grow and upon addition of puromycin they will burst. This may cause the death of infected organoids.

(xxvii) If you have transduced mouse organoids, remove Wnt3a and ROCK inhibitor from the medium when the fragments have formed cystic organoids that reach full size. Continue supplementing with puromycin (1µg/ml) for continued selection. If you have transduced human organoids, retain Wnt3a and ROCK inhibitor throughout the selection period.

(xxviii) Approximately 1 week after starting selection, passage the organoids according to steps 7 to 16 and overlay with the appropriate medium supplemented with puromycin (1µg/ml). **ΔCRITICAL STEP** For continued culture keep organoids in puromycin-containing medium as this prevents silencing of transgenes. If transducing liver organoids, you may proceed to Box 4 if you wish to differentiate the cells to hepatocytes.
? TROUBLESHOOTING

Analysis of organoids

18| To analyze organoid growth and effects of genetic manipulation follow option A to isolate DNA; option B to isolate RNA or option C for organoid staining.

(A) DNA isolation ● TIMING 40min setup, followed by overnight incubation

(i) Culture the organoids as described in steps 7-16 for 5-7 days before collecting organoids as described in step 8. Use at least 1 confluent well (24-well plates) or 2 confluent wells (48-well plates)

(ii) Centrifuge the tube at 300*g* for 5min at 4°C.

(iii) Aspirate the supernatant and resuspend organoids in 15ml of cold Basal medium.

(iv) Repeat steps *18(A)* (ii)-(iii) twice to remove as much of the old basement matrix as possible.

(v) Aspirate the supernatant and resuspend the organoids in 1ml of cold PBS-1X. Transfer the suspension to a 1.5ml centrifuge tube.

(vi) Centrifuge the tube at 300g for 5min at 4°C.

(vii) Aspirate the supernatant completely and add 30µl per sample of Direct-PCR solution supplemented with 0.5µl of Proteinase K (20mg/ml stock).

(viii) Incubate the samples at 60°C overnight.

(ix) Centrifuge the tube at ≥8000g for 10min at room temperature.

(x) Store the DNA at 4°C. **ΔCRITICAL STEP** Storage in lysis buffer (Direct-PCR solution) for a long period may affect the DNA and subsequent PCR amplification. Therefore either use freshly isolated DNA or, if DNA concentration is high enough, dilute the DNA in 270µl distilled H_20 after step *18(A)* (ix).

(xi) If high purity DNA is required (e.g. for DNA sequencing) after step *18(A)* (v) use a DNA purification kit following the manufacturer's instructions

(B) RNA isolation ● TIMING 90min (increases with increased number of samples)

(i) Culture the organoids as described in steps 7-16 for 5-7 days before aspirating the supernatant. Use at least 1 confluent well (24-well plates) or 2 confluent wells (48-well plates). **ΔCRITICAL STEP** Incomplete removal of the cell culture medium may inhibit cell lysis and dilute the lysate, affecting the environment for the binding of RNA to the RNeasy membrane and potentially reducing RNA yield.

(ii) Add 350µl of Buffer RLT (RNeasy Mini kit) per well, collect lysate into a 1.5ml tube and vortex vigorously for 30s to mix.

(iii) To homogenize the lysate, pass the lysate at least 5 times through a blunt 20-gauge needle (0.9mm diameter) fitted to an RNase-free syringe **ΔCRITICAL STEP** Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column.

■**PAUSE POINT** The lysate can be stored at -80°C for 1month.

(iv) Follow the RNeasy Mini kit instructions "Protocol: Purification of Total RNA from Animal Cells" detailed in the RNeasy Mini Handbook.

(C) Staining organoids ● TIMING 1.5-2h for washing organoids, 1h for fixing organoid, 4-5 h and overnight incubation for paraffin embedding or 3-4 days for whole mount staining

i. Use 1 well (24-well plates) or 2 wells (48-well plates) of 80-90% confluent organoids (100-200 organoids). **ΔCRITICAL STEP** Do not overgrow the organoids, as it is easier to conserve the shape of the organoids by using small organoids for this protocol.

ii. Coat a 7ml plastic transfer pipette with PBSB by pipetting the solution up/down for 1-2min. **ΔCRITICAL STEP** PBSB contains BSA or FBS to prevent the organoids attaching to the plastic surface.

iii. Remove medium and add ~1ml cold PBSB.

iv. Gently suspend the basement matrix in the cold PBSB with the coated pipette and carefully transfer the organoid suspension to a 15ml centrifuge tube with the coated pipette.

v. Gently add cold PBS-1X to the top, and allow the organoids to settle under gravity on ice (10min).

vi. Remove the PBS-1X **ΔCRITICAL STEP** Be careful not to aspirate the organoids, which will be only loosely attached to the tube wall.

vii. Add 13ml of cold PBS-1X and incubate on ice for 30min. To wash the basement matrix off, invert the tube 5-10 times or gently pipette up and down 3-5 times with the coated pipette during the 30min incubation.

viii. Allow the organoids to settle under gravity. Carefully remove the PBS-1X.

ix. Repeat the wash steps 18*(C)* (vii)-(viii) for 1-3 times **ΔCRITICAL STEP** Wash steps are important to remove the basement matrix, which can interfere with staining.

x. Add 4ml of freshly prepared fixative to organoids and incubate for 30min on ice.

xi. Gently remove the fixative and replace it with 10ml cold PBS-1X. Let the tube stand on ice for 10min to allow the organoids to settle under gravity. Carefully aspirate the PBS-1X. **ΔCRITICAL STEP** Be careful not to aspirate the organoids.
? TROUBLESHOOTING

xii. Repeat wash step 18*(C)* (xi) twice.

xiii. For whole mount staining store material in PBS-1X at 4°C and skip to step 18*(C)* (xxv) for further instruction. For paraffin embedding, replace the final PBS-1X re-suspension with 70% Ethanol and store at 4°C and continue to step 18*(C)* (xiv) for further instruction.
■**PAUSE POINT** Organoids can be stored at 4°C for weeks.

xiv. **Paraffin embedding** The day before embedding, pre-warm 1 flat-bottom glass tube containing ~3ml of Histowax per sample; 1 plastic transfer pipette per sample and 1 small metal base mould per sample to 65°C in an incubator.

xv. Dehydrate and stain organoids in 0.5% Eosin dissolved in 96% ethanol. Stain for at least 30min. **ΔCRITICAL STEP** Make sure the eosin solution reaches the bottom of the tube by flicking the tube.

xvi. Dehydrate the organoids in 100% ethanol (3 washes of ~30min each).

xvii. Clear organoids using Xylene (3 washes of ~30min each). **ΔCRITICAL STEP** Replace Xylene with n-Butanol if X-gal staining is required.

xviii. Gently transfer organoids to the 5ml flat-bottom glass tube containing the pre-warmed Histowax using a plastic transfer pipette. **!CAUTION** Xylene is a very toxic compound, so avoid co-transferring a large amount of it with the organoids.

xix. Impregnate the organoids in Histowax at 65°C overnight.

xx. Embed the organoids by transferring them to a pre-warmed small metal base mould, using a pre-warmed plastic transfer pipette. Move the organoids gently into the middle of the mould using pre-warmed fine forceps. **ΔCRITICAL STEP** Histowax will solidify at room temperature, so it is very important to work quickly with the pre-warmed material.

xxi. Transfer the mould to a cold area; the Histowax will solidify in a thin layer, which holds the organoids in position.

xxii. Add a tissue cassette on top of the mould as a backing, press firmly and add enough hot Histowax paraffin to cover the face of the plastic cassette.

xxiii. Harden the paraffin on a cold plate and remove the mould.

■PAUSE POINT Blocks can be stored at room temperature for years.

xxiv. Cut sections using a standard microtome and perform standard histological techniques.

xxv. **Whole mount staining** After fixing the organoids (step 18*(C)* (xiii)), remove the PBS-1X and replace with 4ml PBSDT blocking solution. Incubate organoids with gentle agitation for 1-3h at room temperature. Let the tube stand for 10min afterwards to settle the organoids under gravity.

xxvi. Incubate organoids standing at room temperature with the Universal blocking solution with casein (diluted 1:10 from stock in distilled water) for up to 10min. **ΔCRITICAL STEP** This additional blocking step is optional depending on the primary antibody being used.

xxvii. Gently remove the blocking solution and add primary antibody diluted in PBSDT blocking solution OR PBS-1X + 0.1% BSA OR PBS-1X + 0.1% FBS. The best diluent requires optimization based on the antibodies to be used. Incubate for 24-48h with gentle agitation at 4°C.

xxviii. Let the organoids settle under gravity for up to 5min and remove the supernatant. Then, wash the organoids by adding 4ml PBS-1X + 0.1% BSA, incubating at room temperature for 10min.

xxix. Let the organoids settle under gravity and remove the supernatant.

xxx. Repeat the wash steps *18(C)* (xxviii)-(xxix) four times.

xxxi. Incubate organoids with secondary antibodies diluted 1:250 in PBS-1X + 0.1% BSA for at least 2h at room temperature with gentle agitation.

xxxii. Let the organoids settle under gravity and wash off the secondary antibody by adding PBS-1X + 0.1% BSA. Incubate at room temperature for 10min.

xxxiii. Let the organoids settle under gravity before removing the supernatant.

xxxiv. Repeat the wash steps *18C* (xxxii)-(xxxiii) four times.

xxxv. If nuclear staining is required, incubate organoids with a nuclear dye (e.g. Hoechst or DAPI) diluted 1:2000 in PBS-1X for 15min.

xxxvi. Wash organoids by adding PBS-1X, incubating with gentle agitation at room temperature for 10min, letting the tube stand for 10min and gently removing the supernatant.

■PAUSE POINT The immunostained organoids can be resuspended in PBS-1X and stored at 4°C with light protection.

ΔCRITICAL STEP At this point, organoids can be imaged directly in suspension in PBS. However, it is recommended to immediately mount the organoids to a glass slide using an agent such as Vectashield in order to preserve the fluorescence of the secondary antibody conjugates.

xxxvii. Mount organoids to a glass slide using a protein-coated plastic Pasteur pipette (see step 18*(C)* (ii)) in order to minimise damage to the organoids. Remove excess PBS-1X and add an appropriate amount of Vectashield. Add a glass coverslip to the top of the slide and leave it to settle overnight at 4°C with protection from light. Seal the slide and coverslip the next day following standard procedures and image when seal is dry.

TROUBLESHOOTING

See Table 1 for troubleshooting guidance.

Table 1 Troubleshooting table

Step	Problem	Possible reason	Solution
Isolation and single cell dissociation			
1|(A)(ix)	Low duct yield after tissue dissociation	Over- or under-digestion of tissue. Ducts often confused with blood vessels. In case of human pancreas samples, low number of ductal structures in the initial biopsy	Check each fraction for ducts during isolation, comparing to Figure 2. Use Figure 2 as a guide to how ducts look. Start with as much tissue as possible.
1|(B)(viii)	Low cell yield after liver dissociation	Over- or under-digestion of tissue.	Frequently check progress of digestion using a brightfield microscope. The digestion should not exceed 1.5h.
Box|1 step 10	Low cell yield after sorting	Incomplete dissociation or over-digestion. Insufficient mechanical dissociation of the organoids. No cells obtained after dissociation.	Frequently check progress of trypsinization using a brightfield microscope. Pipette a greater number of times both before and after incubation with TrypLE. Confirm that a cell pellet forms after centrifugation. Centrifugation should be performed at 400-500g for at least 5min.
6|	Low colony formation efficiency	Cell death after seeding. Cell viability compromised due to over-digestion	Ensure that Wnt, Noggin and ROCK inhibitor [Y-27632] are added to the Isolation medium, if appropriate. Check cell viability before seeding. Avoid over-digestion by checking fractions constantly.
Passaging and processing			
12|	Loss of organoids	Aspiration of organoids.	Use a low vacuum strength and take care, as BM droplets are only loosely attached to the well or might even be completely detached from the plate. BM will also result in formation of a loose cell pellet after centrifugation. Use a pipette rather than the aspirator to remove the last remaining medium from cell pellets.

		No clear pellet visible or a smear on the wall.	Organoids and BM will sink to the bottom if the tubes are left standing for a while. Mix by pipetting and spin again. Increasing the *g* up to 300*g* can also help.
13|	BM solidifies in tip	BM has warmed in the tip during seeding.	Keep BM on ice as much as possible. Seed BM:cell mixture as soon as cells have been resuspended in BM.
	Air bubbles in the BM	Pipetting the BM too quickly.	To avoid bubbles, pipette slowly and only push to the first stop of the pipette to dispense the BM:cell mixture. If many bubbles are formed, tubes could be centrifuged at 4°C in order to push bubbles to the top of the solution.
16|	Cells attach to the plate	BM spreading to walls of well.	Seed cells in a droplet of BM in the centre of the well and allow to solidify at 37°C without touching the walls of the well. Thaw BM overnight from stocks at 4°C to keep it cold and pre-warm plate to 37°C.
	Low average number of organoids	Insufficient mechanical dissociation of the organoids.	Pipette a greater number of times using the 200µl pipette or narrowed glass Pasteur pipette.
	Inconsistent density of organoids between wells	Organoids will sink to the bottom if the tubes are left standing for a while.	To achieve a consistent density of organoids when seeding, pipette up and down a few times prior to seeding. Pipetting will allow even mixing before seeding.
18|(C) (xi)	Fixed organoids do not settle under gravity	Fixation of organoids in methanol.	When methanol is used as a fixative, organoids will stay in suspension in PBS. Spinning at 10-50*g* for 2-5min will resolve this.
Transient transfection and retroviral transduction			
17|(A)(xv) and 17|(B)(xxv)	Low cell yield	Incomplete dissociation or over-digestion.	Frequently check progress of trypsinization using a brightfield microscope.
		Insufficient mechanical dissociation of the organoids.	Pipette a greater number of times both before and after incubation with TrypLE.
		No cells obtained after dissociation/transfection/transduction.	Confirm that a cell pellet forms after each centrifugation step.
17|(A)(xvi)	No organoids survive the	Poor survival of the transfected cells.	Make sure that the medium is supplemented with ROCK inhibitor [Y-

	selection process		27632] throughout the selection period.
		Low transfection efficiency.	Include a transfection control (a plasmid expressing a fluorescent protein) to confirm that the transfection efficiency is adequate. Increase the number of organoids used for transfection. As with cell lines, the transfection efficiency reduces with increasing passage number. If old organoids are used, a reduced transfection efficiency can sometimes be observed. If human samples are used the transfection efficiency can vary between samples.
17|(B)(xi)	No pellet visible in the viral solution	Inefficient transfection of the Plat-E cells.	Include a transfection control for the Plat-E cells.
		Spinning at too low a speed.	Make sure that the spin speed is 8000*g*.
17|(B) (xxviii)	No organoids survive the selection process	Poor survival of the transduced cells.	Make sure that the medium is supplemented with ROCK inhibitor [Y-27632] throughout the selection period.
		Low transduction efficiency.	Include a transduction control (a plasmid expressing a fluorescent protein) to confirm that the transfection efficiency is adequate. Increase the number of organoids used for transduction.
		Waited too long before starting the selection.	Non-transduced organoids may have a growth advantage compared to transduced organoids. Bursting of large, non-transduced organoids can cause the death of transduced organoids in the immediate vicinity.

TIMING

Steps 1A-6, isolation and seeding of duct cells (mouse liver/pancreas and human pancreas): 4.5h

Steps 1B-6, isolation and seeding of duct cells (human liver): 2.5h

Steps 7-16, passaging organoids for maintenance: 30-40min

Steps 17A, Transfection of organoids: 1.5h preparation of single cells; 20 min preparation of Lipofectamine and DNA; 3-5h transfection; 20-30 min seeding; 2-3h selection by FACS or 1-2 weeks by antibiotic selection

Steps 17B, Transduction of organoids: 5-6d (~1h hands-on time) for transfection of Plat-E cells; 12.5-16.5h for virus collection; 1h for preparation of viral solution and cell suspension; 7-8h (~1h hands-on time) for retroviral transduction; and ~1-2 weeks for selection

Step 18A, Analysis of organoids, DNA isolation: 1d (~40min hands-on time)

Step 18B, Analysis of organoids, RNA isolation: 90min (increases with increased number of samples)

Steps 18C, Analysis of organoids, Staining organoids: 1.5-2h for washing organoids, 1h for fixing organoid, 4-5 h and overnight incubation for paraffin embedding or 3-4 days for whole mount staining

Box 1, Enrichment of duct stem cells by FACS sorting: 2-3h

Box 2, Preparation of Wnt3a and RspoI conditioned medium: 2-3 weeks

Box 3, Cryopreservation and Thawing Organoids: 25min for cryopreservation and 25 min for thawing organoids

Box 4, Differentiation of mouse and human liver organoids: 15d (~4.5h hands-on time)

ANTICIPATED RESULTS

Our protocols are extremely robust and generate reproducible results when tissue dissociation is accurately performed and media composition is precise. Therefore, cell isolation and preservation of recombinant protein stocks are very important. In liver and pancreas organoid cultures, the isolated ducts or sorted ductal cells embedded in Matrigel or BME2 and cultured under the above defined culture conditions quickly self-organize and generate 3D structures (organoids) with 90-100% efficiency (when starting with duct structures) and 15-30% efficiency (when starting with sorted cells; Fig. 2A, liver; 2B, pancreas). 1-2 days after seeding the duct structures or sorted cells start proliferating to generate a cyst-like structure that, in ~1 week (duct structures) or 2 weeks (sorted cells), can be mechanically dissociated and split in a 1:4-1:6 ratio for further expansion (Fig. 2A, liver; 2B, pancreas). Typically, liver and pancreas organoids are formed by a single-layer epithelium of cuboidal cells expressing ductal markers (e.g. Krt19) surrounding a central lumen. Liver organoids also exhibit some areas of pseudo-stratified epithelium (Fig. 3B) that resembles a liver bud structure and express low levels of the hepatocyte marker HNF4α[8].

Culturing in hepatocyte differentiation medium, the cuboidal single-layer ductal epithelium becomes a stratified polygonal epithelium formed by Albumin-positive (ALB^+) and HNF4α-positive cells that fill the original lumen (Fig. 3C). Generally, as determined by FACS or immunohistochemistry analysis, ~40% (mouse) or ~60% (human) of cells differentiate into ALB^+, $HNF4\alpha^+$ cells at day 11-14 after initiation of differentiation. Some of the cells (~1%) become bi-nucleated, a hallmark of mature hepatocytes. Following long-term exponential expansion, the cultures remain genetically stable with virtually no accumulation of base substitutions as determined by WGS analysis[9]. Following expansion, characterization of the cultures can be performed using standard RNA, protein or DNA techniques (Fig. 1) similar to a standard cell line.

For genetic manipulation, organoid cultures can be transiently transfected or virally transduced using the protocols described above (Fig. 4). To achieve high levels of transfection (~10%) or transduction (~50%) efficiency, ensure the organoids have reached their maximum size but have a minimum amount of dead cells accumulated in their lumens prior to genetic manipulation (see representative Fig. 5A). Then, when preparing single cells, it is important to balance the number of single cells, larger fragments and viability of the fragments or single cells. Incubating for too long in TrypLE reduces the recovery of single cells, therefore allowing some larger fragments will increase the overall viability (see representative Fig. 5B). Following centrifugation and incubation, very small cystic or round structures may appear. This confirms that the TrypLE treatment was adequate and ~10-30 organoids perwell should be expected to grow out over the following days. Where a plasmid expressing a fluorescent protein has been used, we observed that fluorescence was visible after 36-48 hours (Fig. 5C) peaking at 48h after transfection and decreasing significantly after 96 hours. When sorting for GFP signal was used for selection, antibiotic selection was avoided. Alternatively, when GFP selection is not available, an appropriate antibiotic selection is required. The timing for the antibiotic selection depends on the antibiotic used. When using puromycin as the selection agent, significant death of non-transfected/non-infected organoids was observed as soon as 4-5 days after transfection/transduction. Alternatively, when hygromycin was used, the selection process was slower and took up to 8 days. Whenever possible, transduction efficiency should be confirmed by the detection of a reporter (e.g., dsRed expression if using Addgene 32702, 32703 or 32704

retroviral vectors). Alternatively, when no reporter marker is available, a transduction control (usually a constitutive GFP-expressing virus) can be used to determine the transduction efficiency (Fig. 5D).

FIGURE LEGENDS

Figure 1. Schematic representation of organoid isolation, culture and characterization. The relevant steps of the protocol for each section are highlighted in red. Ducts or single cells can be isolated from human or mouse liver or pancreas (upper steps) and cultured to form 3D organoids (representative brightfield images of each species and tissue type shown 5-7 days following passaging). Following expansion, organoids can be characterized using standard molecular biology techniques (bottom right steps) or frozen for storage (bottom steps). Scale bar for organoids in expansion represent 200um. UK NHS REC and Home Office permission was obtained for the use of human and mouse samples, respectively.

Figure 2. Examples of isolated ducts and growing organoids. Brightfield images of the isolation and growth of organoids from mouse liver (A) and pancreas (B) using whole ducts (upper panels) and single cells (lower panels). For both tissues, ducts increase their lumen volume after 2 days in culture and single cells will form full organoids after 6-7 days in culture. Organoids can then be expanded long term (after passaging pictures were taken 3 months after isolation). Scale bars represent 100µm for the ducts (days 0 and 2), 25µm for the single cells (days 0, 6 and 7) and 500µm for organoids after passaging. UK NHS REC and Home Office permission was obtained for the use of human and mouse samples, respectively.

Figure 3. Differentiation of liver organoids. (A) Scheme indicating the different steps and medium used during isolation, expansion and differentiation. Representative brightfield images showing human liver organoids at the different stages (left panel, 2 days following isolation; centre panel, 5 days following passaging; right panel, 15 days from the start of the differentiation protocol). Scale bars in brightfield images represent 100µm (IM) and 500µm (EM, DM). (B) Representative images of mouse (left panels) and human (right panel) liver organoids cultured under expansion conditions for 6 days following passaging and stained for the ductal markers MIC1-1C3 and Sox9 (upper left panel), EpCAM (upper right panel) and E-Cadherin (lower left panel). Typically, liver organoids will self-organize into a single-layered epithelial compartment (arrow, lower left panel) and a pseudo-stratified compartment. Scale bars represent 100µm. (C) Representative images of mouse (left panel) and human (right panel) liver organoids cultured under differentiation conditions for 15 days and stained for the differentiated hepatocyte markers HNF4α (red, left panel) and Albumin (green, left; red, right), and the tight junction marker ZO-1 (green, right panel). See Supplementary Table 2 for information on antibodies used. Scale bars represent 25µm (left) and 50µm (right). UK NHS REC and Home Office permission was obtained for the use of human and mouse samples, respectively.

IM, Isolation medium; EM, Expansion medium; DM, Differentiation medium; ECAD, E- Cadherin; ALB, Albumin; EpCAM, Epithelial cell adhesion molecule

Figure 4. Scheme summarizing the relevant steps in organoid transduction (left) and transfection (right) protocols. First steps deal with the production of virus using PlatE cells (Transduction) and formation of DNA:lipofectamine complexes (Transfection) in order to introduce transgenes to the organoids. Both protocols require the dissociation of organoids to single cells followed by spin-inoculation before cells can be seeded and organoids derived in a clonal manner.

Figure 5. Transfection and transduction of liver organoids. (A) Representative image of mouse liver organoids indicating the optimal confluence for the day of transfection/transduction, ~6 days following passaging. Scale bar 2mm. (B) Brightfield image showing the optimal cells and cell fragments size for transfection/transduction. Scale bar 400µm. Example shows mouse cells (C) Representative fluorescence image showing mouse liver organoids transduced with a viral vector expressing GFP two days after transfection. Scale bar 400µm. (D) Representative fluorescence image

showing human liver organoids transfected with a GFP-expressing vector one day post-transfection. Scale bar 1mm.

Supplementary Table 1. Media composition summary. Table summarising the components of the media described in this paper. Suitable stock concentrations and dilutions are also described. Reagents and/or concentrations highlighted in green differ between human and mouse media, reagents and/or concentrations highlighted in orange differ between expansion media (EM) and differentiation media (DM), reagents and/or concentrations in red differ between liver and pancreas organoid media.

Supplementary Table 2. Antibody details. Table summarising the antibodies used in Figure 3.

Box1| Enrichment of duct stem cells by FACS sorting ● TIMING 2-3h

1 Resuspend the ductal structures in 5ml of pre-warmed TrypLE solution supplemented with 5µl of DNaseI (10mg/ml). After addition of the TrypLE solution use a narrowed glass Pasteur pipette (obtained by flaming a glass Pasteur pipette to make the aperture thinner to ~1/4 of original aperture size) and pipette up and down 5 times. Incubate at 37°C for 5-10min.

2 Check the TrypLE digest solution every 2 min using a brightfield microscope. Stop the digestion when the majority (90-95%) of material consists of single cells (see Fig. 2 for example). ΔCRITICAL STEP Do not over-digest the material.

3 Add 10ml cold Wash medium to stop the digestion and pellet the material by centrifuging at 300-400*g* for 5min at 8°C. Discard supernatant and add 10ml Wash medium, resuspending the pellet, before pelleting the material again by centrifuging at 500*g* for 5min at 8°C to wash out any remaining TrypLE.

4 Discard supernatant and resuspend pellet in 5ml of Wash medium. Filter the resuspended material into a 50ml centrifuge tube through a 40µm filter. This process should leave only single cells in the centrifuge tube. Count the number of cells using a standard haemocytometer or similar.

5 Divide the material into a negative control and a sample for antibody staining, transferring 10% and 90% of the material respectively into suitable FACS tubes. Pellet the cells by centrifuging at 400*g* for 5min at 8°C.

6 Resuspend the pellets in 2ml blocking solution: DMEM/Glutamax with 2% FBS, 2µl DNaseI (10mg/ml) and 10µM ROCK inhibitor [Y-27632], and incubate on ice for 10-20min.

7 Pellet the cells by centrifuging at 400*g* for 5min at 8°C. Discard the supernatant, resuspend the pellet in 500µl of Wash medium supplemented with 0.5µl DNaseI (10mg/ml) and 10µM ROCK inhibitor [Y-27632]. For sample tubes undergoing staining add the desired antibody and incubate tubes on ice in the dark for 1h. ● TIMING using conjugated antibodies is desirable as it reduces incubation time and increases cell viability.

8 Add 3ml Wash medium and pellet cells by centrifuging at 400*g* for 5min at 8°C.

9 Discard the supernatant and repeat step 8. Resuspend the pellet in 500µl Wash medium supplemented with 10µM ROCK inhibitor [Y-27632].

10 Go to the FACS sorter and following gate separation sort the desired cells into a collecting tube containing culture medium supplemented with 10µM ROCK inhibitor [Y-27632].
? TROUBLESHOOTING

11 Pellet the sorted material by centrifuging at 500*g* in a table top microcentrifuge for 5min at 8°C. Seed the cells as described in the step 2 of the main protocol.

END BOX

Box2| Production of Wnt3a or Rspol conditioned medium ● TIMING 2-3 weeks

Reagents

- BD Falcon™ 150cm² Tissue Culture Flask, vented cap (BD cat.no. 355001)
- 500cm plates (Corning cat.no. 734-1727)
- 50ml conical centrifuge tubes (SLS cat.no. 352070)
- 500ml filter cups (Millipore cat.no. SCGPS05RE)
- DMEM, high glucose, GlutaMAX, pyruvate (Life Technologies cat.no. 31966-021)
- FBS (Life Technologies cat.no.26010066)
- Penicillin/Streptomycin (10,000 U/mL) (Life Technologies cat.no. 15140-122)
- Advanced DMEM/F12 (Life Technologies cat.no. 12634-010)
- GlutaMax (100X) (Life Technologies cat.no. 35050-068)
- HEPES 1 M (Life Technologies cat.no. 15630-056)
- TrypLE (Life Technologies, cat.no. 12605-028)
- Zeocin™ (Thermofisher, cat.no. R25005)

Wnt3a conditioned medium

Growth medium: DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin and 300µg/ml Zeocin. Store at 4 °C for up to one week.

Harvest medium: DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin. Store at 4 °C for up to one month.

1 Plate 1.5-2×10^6 cells of the L-Wnt3a cell line into a T150 flask in 35ml of growth medium, pre-warmed to 37 °C.

2 Expand cells in growth medium by passaging when cells are at ~75% confluence. Passage by removing medium and incubating in 3ml TrypLE, pre-warmed to 37 °C. Incubate for 2-3min until all cells are in suspension, then add ~5ml of growth medium to the flask. Pool cells and re-seed 1.5×10^6 cells per T150 flask in 35ml of growth medium as in step 1.

3 When there are 20-30 T150 flasks each at ~75% confluence, passage cells as in step 2 but re-seed 500cm plates with 4.5×10^6 cells per plate in 100ml of growth medium.

4 When plates are ~70% confluent then change growth medium to 100ml harvest medium per plate. Incubate in this medium for 1 week

5 Remove medium into 50ml tubes. Centrifuge at 500*g* for 5min to remove cells.

6 Filter medium using 500ml filter cups. Mix and aliquot into 25ml aliquots. Wnt3a conditioned medium can be stored at 4 °C for up to 6 months.

Rspol conditioned medium

Growth medium: DMEM supplemented with 10% FBS and 150µg/ml Zeocin. Store at 4 °C for up to one week.

Harvest medium: Advanced DMEM/F12 supplemented with 1% Penicillin/Streptomycin, 1% Glutamax, and HEPES 10mM. Store at 4 °C for up to one month.

1 Plate 1.5-2×10^6 cells of the L-Wnt3a (Wnt3a conditioned medium) or 293T-HA-RspoI-Fc (RspoI conditioned medium) cell lines into a T150 flask in 35ml of growth medium, pre-warmed to 37 °C.

2 Expand cells in growth medium by passaging when cells are at ~75% confluence. Passage by removing medium and incubating in 3ml TrypLE, pre-warmed to 37 °C. Incubate for 2-3min until all cells are in suspension, then add ~5ml of growth medium to the flask. Pool cells and re-seed 1.5×10^6 cells per T150 flask in 35ml of growth medium as in step 1.

3 When there are 20-30 T150 flasks each at ~75% confluence, change growth medium to 35ml harvest medium per flask. Incubate in this medium for 1 week

4 Remove medium into 50ml tubes. Centrifuge at 500*g* for 5min to remove cells.

5 Filter medium using 500ml filter cups. Mix and aliquot into 5ml aliquots. RspoI conditioned medium can be stored at -20 °C for up to 6 months.

END BOX

Box3| Cryopreservation and Thawing Organoids ● TIMING 25min for cryopreservation and 25 min for Thawing organoids

Freezing organoids. Pre-cool a freezing container at 4°C.For each cryovial tube you will need at least 1 confluent well (24-well plates) or 2 confluent wells (48-well plates) to have been grown prior to freezing.

1 Proceed as described in steps 8 to 12 of the main protocol, then gently resuspend the cell aggregates in 500µl of cold freezing medium per 1 well (24-well plates) or 2 wells (48-well plates). Then transfer the suspension to cryovials (500µl each) and place them in the pre-cooled cell freezing container. Transfer to -80°C. ΔCRITICAL STEP The freezing medium contains DMSO, which is toxic for the cells at room temperature, so use cold freezing medium and do not exceed a total of 5min between adding the freezing medium to the cells and transferring them to -80°C.

2 Following a minimum of 24h at -80°C, cryovials can be transferred to liquid nitrogen for long-term storage.

■**PAUSE POINT** Cells can be stored in liquid nitrogen for long-term storage (1-2 years).

Thawing organoids

3 Warm a water bath to 37°C and prepare a 15ml tube with 10ml of Basal medium. Pre-warm this medium to 37°C. Pre-warm an appropriate 24-well plate.

4 Incubate the cryovial in the 37°C water bath, stopping when the ice is almost completely thawed. Transfer the thawed cell aggregates to the pre-warmed 15ml centrifuge tube containing Basal medium.

5 Centrifuge the tube at 200g for 5min at 8°C.

6 Aspirate the supernatant completely.

7 Proceed as described in steps 13 to 14 of the main protocol, and overlay with the appropriate Expansion medium (500 µl per well [24-well plates] or 250 µl per well [48-well plates]) supplemented with 10µM of ROCK inhibitor [Y-27632].

8 Fully change the medium every 3-4 days. ROCK inhibitor can be removed after 6 days in culture.

END BOX

Box4| *In vitro* hepatocyte differentiation ● TIMING 15 days

ΔCRITICAL STEP ***This procedure is only performed on liver organoids.***

1. If you wish to differentiate the cells within the liver organoids to hepatocytes, follow option A if working with mouse organoids and option B if working with human liver organoids.

(A) Mouse liver organoids

(i) Seed organoids as previously described in steps 7 to 15 of the main protocol (day 0), and culture using mouse liver expansion conditions for 3 days.

(ii) Change the medium to mouse Differentiation medium (day3). Replace with fresh Differentiation medium every day for up to 9 days (day 12). At this stage cells already express mature hepatocyte markers such as Albumin or Cyp3A.

(iii) Supplement Differentiation medium with 3µM Dexamethasone from day 13 to day 15. Replace medium with fresh, fully supplemented medium every day until day 15 after which organoids are ready to be processed for analysis (step 18 of the main protocol).

(B) Human liver organoids

(i) Seed organoids as previously described in steps 7 to 15 of the main protocol (day 0) and culture using human liver expansion conditions supplemented with BMP7 (25ng/ml) for 5 days.

(ii) After 5 days, change the medium to human Differentiation medium. Replace with fresh Differentiation medium every 3 days for up to 10 days (day 15) after which organoids are ready to be processed for analysis (step 18 of the main protocol).

END BOX

AUTHORS' CONTRIBUTIONS

MH and SFB, developed the culture system for liver and pancreas. BKK developed the genetic manipulation of organoid cultures. HC supervised the development of organoid cultures and their genetic manipulation. LB, AAR, CH prepared figures. LB, AAR, CH, SFB and MH wrote the manuscript. All authors commented on the manuscript.

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COMPETING FINANCIAL INTERESTS

The authors declare no financial competing interests. MH, SFB and HC are inventors on patent applications on organoid cultures.

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