Establishment of human fetal hepatocyte organoids and CRISPR-Cas9-based gene knockin and knockout in organoid cultures from human liver

Hendriks, D.; Artegiani, B.; Hu, H.L.; Lopes, S.C.D.; Clevers, H.

nature protocols

PROTOCOL

https://doi.org/10.1038/s41596-020-00411-2

Check for updates

Establishment of human fetal hepatocyte organoids and CRISPR–Cas9-based gene knockin and knockout in organoid cultures from human liver

Delilah Hendriks[1,2,5]✉, Benedetta Artegiani[1,2,3,5]✉, Huili Hu[1], Susana Chuva de Sousa Lopes[4] and Hans Clevers[1,2,3]✉

The liver is composed of two epithelial cell types: hepatocytes and liver ductal cells. Culture conditions for expansion of human liver ductal cells in vitro as organoids were previously described in a protocol; however, primary human hepatocytes remained hard to expand, until recently. In this protocol, we provide full details of how we overcame this limitation, establishing culture conditions that facilitate long-term expansion of human fetal hepatocytes as organoids. In addition, we describe how to generate (multi) gene knockouts using CRISPR–Cas9 in both human fetal hepatocyte and adult liver ductal organoid systems. Using a CRISPR–Cas9 and homology-independent organoid transgenesis (CRISPR-HOT) approach, efficient gene knockin can be achieved in these systems. These gene knockin and knockout approaches, and their multiplexing, should be useful for a variety of applications, such as disease modeling, investigating gene functions and studying processes, such as cellular differentiation and cell division. The protocol to establish human fetal hepatocyte organoid cultures takes ~1–2 months. The protocols to genome engineer human liver ductal organoids and human fetal hepatocyte organoids take 2–3 months.

Introduction

The liver is a vital organ with many important functions, including central roles in production and storage of amino acids and vitamins, metabolism and detoxification. Different in vitro cultivation methods and culture conditions to maintain non-proliferative primary liver cells have been recently developed that allow mature hepatocyte phenotypes to be retained for 2–4 weeks in culture[1–4]. In addition, optimized protocols are continuously being developed to terminally differentiate human embryonic or induced pluripotent stem (ES/iPS) cells into hepatocyte-like or hepatobiliary-like organoids[5–8]. These iPS-derived hepatocyte-like cells further develop into human liver buds upon transplantation in mice[5,9]. In addition, direct conversion of human fibroblasts into hepatocyte-like cells has been proven possible by manipulating expression of key transcription factors involved in guiding hepatocyte fate and differentiation[10–12]. Although constituting important achievements, these methods do not allow expansion of (primary) human liver cells. The inability to expand (primary) human liver cells long term limits their use in chronic disease modeling and limits genome engineering to viral-based approaches[13], which cannot be used to make precise gene modifications.

A few years ago, our group established culture conditions that allowed the long-term expansion and maintenance of human adult EpCAM$^+$ biliary epithelial cells as organoids (human liver ductal organoids)[14]. In expansion medium, these organoids display a biliary phenotype, but they can also be induced to differentiate into hepatocyte-like cells, at which stage they lose their capacity to proliferate[14]. The expansion of primary human hepatocytes (PHHs) has proven challenging. Recent achievements have included strategies in which, starting from PHH, cells are expanded through a progenitor-like intermediate stage. The cells subsequently acquire mature hepatocyte phenotypes

[1]Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences, Utrecht, the Netherlands. [2]Oncode Institute, Utrecht, the Netherlands. [3]The Princess Maxima Center for Pediatric Oncology, Utrecht, the Netherlands. [4]Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, the Netherlands. [5]These authors contributed equally: Delilah Hendriks, Benedetta Artegiani. ✉e-mail: d.hendriks@hubrecht.eu; b.a.artegiani@prinsesmaximacentrum.nl; h.clevers@hubrecht.eu

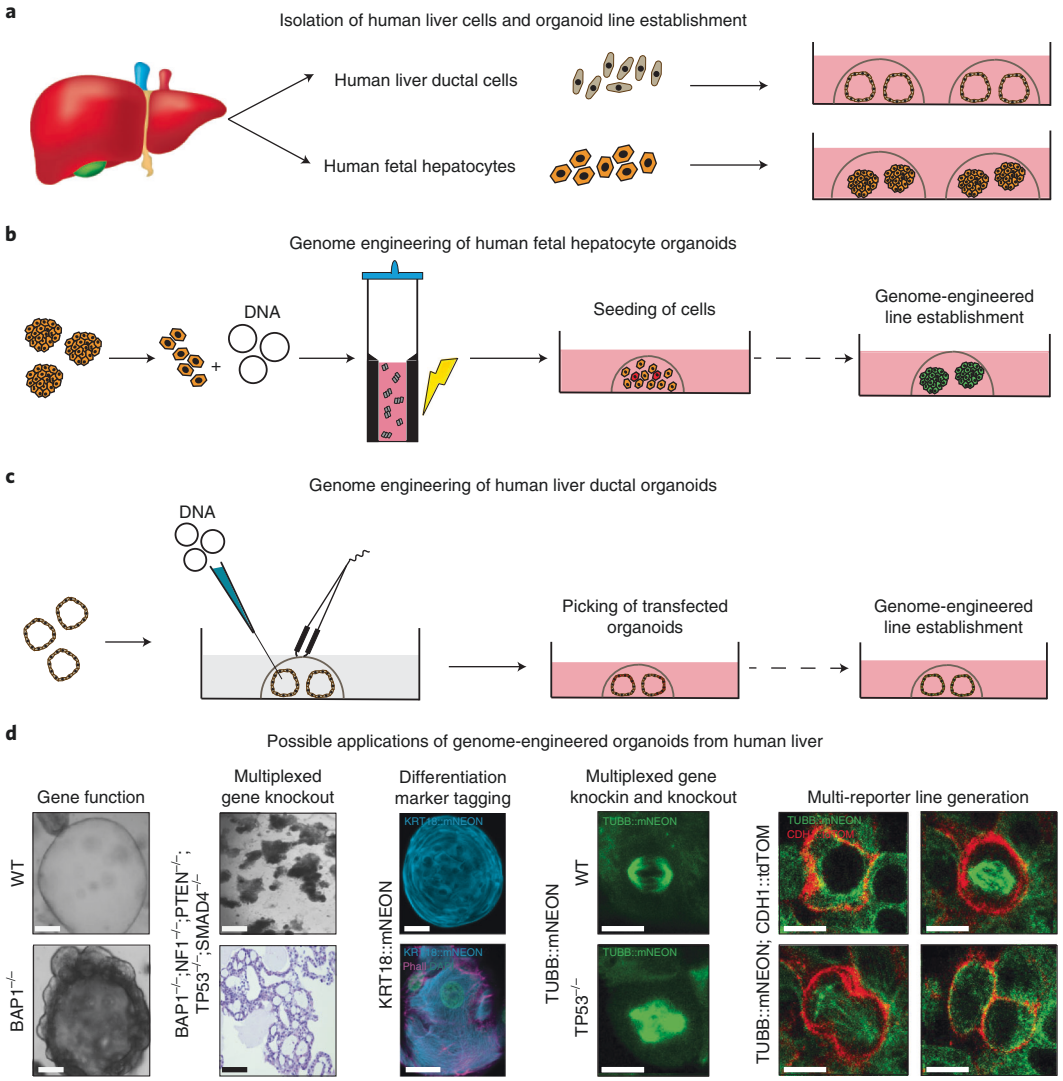


Fig. 1 | Schematic overview of this protocol. a, The two types of epithelial cells (hepatocytes and ductal cells) from human fetal and adult liver can be isolated and used to establish organoid cultures in the respective media. **b**, Human fetal hepatocyte organoids are genome engineered using a cuvette-based electroporation as single cells. **c**, Human liver ductal organoids are genome engineered as intact organoids upon injection of the DNA into the lumen and electroporation using tweezer electrodes. **d**, Examples of possible applications of the genome-engineered human fetal hepatocyte and liver ductal organoids. Images shown in the lower panel are reproduced from Artegiani et al.[22,23]. Scale bars in **d**: from left to right, upper and lower box, 100 μm; upper box, 100 μm; lower box, 300 μm; upper box, 50 μm; lower box, 10 μm; upper and lower boxes, 10 μm.

upon terminal differentiation, induced by further changes in media composition combined with a switch to 3D cultivation[15–18]. Very recently, two studies described long-term expansion of adult mouse hepatocytes as organoids without the need to transdifferentiate them through an intermediate stage[19,20]. In the latter study, culture conditions for long-term maintenance and expansion of primary hepatocyte organoids from human fetal liver were also described[20]. Although of fetal origin, these organoids possess typical hepatocyte features, such as characteristic hepatocyte marker expression and secretion of albumin.

These established human organoid culture systems that allow the expansion of the two epithelial cell types of the liver—the ductal cells and the hepatocytes—open up the possibility of genome engineering primary liver cells and the generation of stably mutant lines. Combining organoid cultures with CRISPR–Cas9 genome engineering leverages the broad applicability of these models to study important biological processes and to model diseases[21]. These genome-engineered organoids helped to elucidate the roles of genes involved in liver cancer through gene knockout[22], as well as understanding modes of human hepatocyte division and hepatocyte ploidy through gene knockin combined with gene knockout[23] (Fig. 1). Here we provide protocols for (i) the long-term expansion of

human fetal hepatocyte organoids and (ii) genome engineering strategies, including gene knockout and gene knockin, and associated applications for both human liver ductal organoids and human fetal hepatocyte organoids (Fig. 1), as first described in refs. [20,22,23].

Development of human fetal hepatocyte organoid cultures

In previous efforts from our group, as well as other groups, culture conditions were established that allow the long-term expansion of healthy adult human stem cell-derived organoids from most organs[14,20,24–28]. Initially, conditions were established for the intestine[24,25], and this was followed by the establishment of additional human organ-specific organoid cultures[14,20,26–28]. Specific conditions were optimized for the different cultures derived from various organs, but all required the embedding and cultivation in extracellular matrix-based gels. As a part of these efforts, culture conditions were developed that allow the long-term expansion of human adult liver ductal cells as organoids[14]. $EpCAM^+$ biliary epithelial cells derived by these conditions[14] can be stimulated to proliferate in culture by stimulation/inhibition of signaling pathways important for liver development and regeneration (including the Wnt, TGFβ and EGF/FGF signaling pathways). The other main epithelial cell type of the liver, the hepatocyte, could, until very recently, not be directly expanded in vitro. However, by tweaking culture conditions, two recent studies demonstrated the ability to culture adult mouse hepatocytes as organoids long term[19,20]. In the study from the Nusse group, TNFα was found to be a critical component required to support long-term expansion of adult mouse hepatocytes. In the study from our group[20], culture conditions were established without the need of TNFα but with inclusion of FGF-7-, FGF-10- and RSPO1-conditioned medium (RSPO1-CM). In addition, CHIR-99021, an inhibitor of GSK3β, part of the β-catenin destruction complex, proved to be essential for hepatocyte expansion (also present in the medium reported by Peng et al.[19]). These findings are consistent with previous observations that strong activation of the Wnt pathway is important to promote hepatocyte regeneration in vivo[29].

The knowledge of appropriate culture conditions for mouse hepatocytes was used to develop conditions that achieve long-term expansion of human fetal hepatocytes[20]. Starting from human fetal liver material, a short and mild collagenase type IV digestion is required to preserve human hepatocyte viability, followed by enrichment of the hepatocytes by low-speed centrifugation steps. Culture conditions remarkably similar to the ones used for the mouse hepatocytes, with the addition of TGFα, are able to promote expansion of human fetal hepatocytes as organoids in vitro. Characterization of this system by immunofluorescence (refer to Fig. 5 in Hu et al.[20]) revealed abundant expression of typical hepatocyte markers. RNA sequencing (both bulk and single-cell) revealed similarities of expression with PHH and identified subpopulations expressing high levels of hepatocyte markers, such as ALB and SERPINA1 (refer to Fig. 6 in Hu et al.[20]). In Supplementary Fig. 1, we show further analysis of this data set[20], demonstrating that the hepatic phenotypes of these organoids are stable during long-term passaging. In Supplementary Fig. 2, we furthermore provide novel data and depict the inter-donor variability with regard to gestational age (10–20 weeks of gestation), which illustrates some similarities in mRNA expression patterns of selected hepatocyte, biliary and hepatoblast markers in the different organoid lines. These human fetal hepatocyte organoids also have superior engraftment in mouse liver in vivo compared to human liver ductal organoids differentiated toward hepatocyte-like cells[14,20].

Development of genome engineering strategies for organoid cultures from human liver

Liver cells are notoriously difficult to transfect in vitro[30]. Broutier et al. reported that liver ductal organoids can be infected with lentiviruses to achieve stable integration of exogenous DNA[31]. However, lentiviruses integrate randomly, and variable copy numbers can be inserted, which does not enable precision genome editing or controlled manipulation. Transfection of human liver ductal cells has also been reported[31], although stable organoid line establishment from these transfected cells was not shown.

We recently reported a method of transfection that relies on manipulation of single intact human liver ductal organoids still embedded within the BME droplet[22]. This method closely resembles and mimics the procedure used for in utero brain electroporation[32]. In this approach, DNA is injected into the lumen of the organoids, after which the entire BME droplet is electroporated using tweezer electrodes. After 2–3 d, positively electroporated organoids (as assessed by co-transfection with a plasmid constitutively expressing a fluorescent reporter) are recovered from the BME droplet and sheared into small organoid pieces. Outgrowing organoids are then dissociated into single cells after

one passage, to achieve outgrowth of clonal lines. Performing electroporation of intact organoids promotes cell survival of the electroporated cells, and we found it to be a crucial step to achieve cellular outgrowth after electroporation and to robustly generate genome-engineered organoid lines.

In contrast to human liver ductal organoids, human hepatocyte organoids are dense structures, so we did not attempt to electroporate using this method. To perform genome engineering of these cultures, we, instead, adapted an electroporation method relying on transfection of the organoids as single cells or small clumps of cells in cuvettes[33]. By optimizing the electroporation settings and single-cell outgrowth conditions (addition of extra ROCK inhibitor to prevent anoikis), we could achieve efficient transfection and subsequent establishment of clonal genome-engineered human fetal hepatocyte organoid lines[23].

Applications of the methods and future possibilities

The establishment of human fetal hepatocyte organoid cultures will greatly facilitate studies of basic human liver biology and, owing to their long-term stability, might also allow modeling of some liver diseases and assessment of drug hepatotoxicity. They could also potentially be used in studies of liver infection. In addition, these organoids further progress ongoing research to optimize liver transplantations for future regenerative medicine applications.

The established and optimized transfection methods for the two organoid systems of the human liver can be used to perform CRISPR–Cas9-mediated genome engineering, including both gene knockin and gene knockout[22,23]. For gene knockout, previous studies introduced frameshifting indels by relying on the non-homologous end joining (NHEJ)-mediated imprecise repair of the CRISPR-Cas9-induced double-strand break (DSB) due to the repeated cutting and re-ligation of the induced break[34–37]. Similarly, this strategy, in combination with our optimized transfection methods in human liver ductal organoids, enabled loss-of-function mutations found in cholangiocarcinoma to be mimicked[22]. In addition to disease modeling, this approach enables interrogation of gene function in human liver, as, for example, revealed for *BAP1* (ref. [22]), and could be used to perform whole-genome CRISPR screens.

Very few reports have been able to show successful generation gene knockin in human organoids[38–41]. The scant existing studies relied on conventional approaches based on homology-directed repair (HDR) and have resulted in generation of a few knockin lines (i.e., tagging of *LGR5* and *KRT20* (ref. [40])) or achieved gene correction (repair of ΔF508 in the *CFTR* gene) in cystic fibrosis intestinal organoids[38]. So far, these studies have been restricted to the intestine, mostly to colorectal cancer organoids. Recently, low efficiency of HDR-mediated gene knockin has been linked to the TP53 damage response upon a Cas9-mediated DSB, and induction of a transient cell cycle arrest in untransformed cells[42,43]. Therefore, novel approaches have aimed at increasing HDR efficiency by transient inhibition of TP53 (ref. [44]). Although most studies have employed HDR owing to its reported preciseness in generating gene knockins[45], few studies have focused on using NHEJ to accomplish precise gene knockin owing to the hypothesis that this pathway is more error prone[46]. However, the few studies in mice and cell lines that have reported the ability to use NHEJ for gene knockin have demonstrated higher efficiencies of knockin than the studies using HDR[47–50]. We recently demonstrated that NHEJ can efficiently and precisely mediate the insertion of exogenous DNA (outperforming HDR by ~ten-fold) in human wild-type organoids[23]. This approach, termed CRISPR-HOT, allows tagging of different constitutively and non-constitutively expressed genes in a variety of human organoid types, including human liver ductal and hepatocyte organoid systems. CRISPR-HOT enables labeling cell type markers, visualizing subcellular structures and studying biological processes. Owing to the robustness of this method, gene knockin can also be easily multiplexed with gene knockout, which further facilitates studies regarding gene function. As an example, we characterized modes of human hepatocyte division in wild-type and TP53 mutation backgrounds[23]. An alternative strategy to express tagged genes is to transduce organoids with viruses[31]. However, the critical limitation of this approach is that the delivered gene is overexpressed and is not controlled by endogenous regulatory elements. In addition, because the integration of the viruses is random, and copy number integration cannot be controlled, unpredictable phenotypes could be produced. Endogenous gene tagging avoids all these limitations and is, therefore, a preferable approach. Using this approach to genetically engineer the two human liver organoid culture systems should further enhance the ability of researchers to answer important biological questions.

Box 1 | Freezing and thawing of human fetal hepatocyte organoids

Procedure ● Timing 1–2 d

▲ **CRITICAL** Human fetal hepatocyte organoid lines can be successfully frozen down and subsequently thawed. We recommend that this be done as soon as enough material is available and when the line has been established (after passage 5).

1 Harvest the organoids in ice-cold wash buffer from the BME in a conical 15-ml tube and perform mechanical disruption into small organoid pieces. We usually disrupt the organoids to a smaller size than the size used for regular organoid passaging (diameter of 10–50 μm). Top up the volume of the tube with ice-cold wash buffer. We recommend freezing at least two wells of a 12-well plate (400,000 cells) to allow for sufficient recovery of the organoids.

2 Centrifuge the tube for 5 min at 400*g* at 4 °C.

3 Aspirate the wash buffer with a glass pipette and resuspend the organoid pellet in an appropriate volume of freezing medium. When freezing two wells of a 12-well plate, we recommend 1 ml of recovery cell freezing medium. Transfer the solution to a sterile cryogenic storage vial.

4 Freeze the cells using a controlled rate freezing apparatus at −70 °C to −80 °C overnight.

5 Transfer the cells to a liquid nitrogen storage container.

■ **PAUSE POINT** Frozen organoids can be stored for at least 2 years in liquid nitrogen.

6 For recovery of frozen organoids, quickly thaw the vials in a 37 °C water bath until the solution is defrosted.

7 Transfer the organoids to a 15-ml conical bottom tube containing 10 ml of wash buffer.

8 Centrifuge the tube for 5 min at 400*g* at 4 °C.

9 Aspirate the medium and resuspend the organoids in an appropriate volume of BME resuspension solution.

10 Plate the organoids as described in Steps 30–33 and subsequently maintain the line as described in Steps 36–43. We recommend plating the cells into the number and size of wells that they were frozen down from.
▲ **CRITICAL** Grow the cells in isolation HEP medium in place of regular HEP medium until organoids of a normal size (for that line) are formed (diameter of >100 μm).

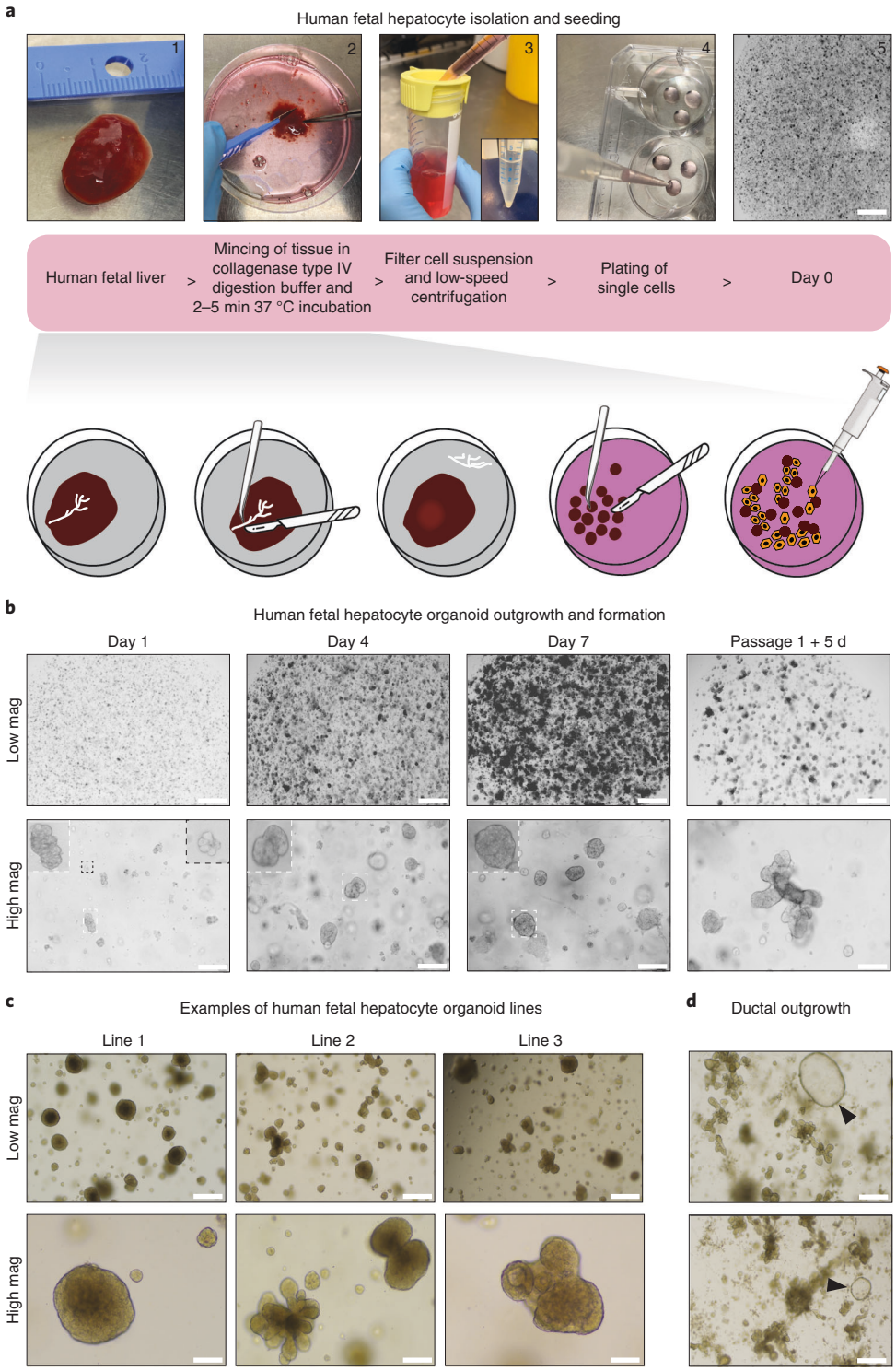
11 Carefully observe the phenotype of the thawed organoids during ongoing passage. The phenotype (morphology and growth speed) should be similar to that seen before cryopreservation. Occasionally, cystic ductal-like organoid structures appear upon outgrowth, which, in our hands, indicates poor recovery of the line. In such cases, we recommend thawing another vial.
? TROUBLESHOOTING

Experimental design

In the current protocol, we outline experimental procedures for the establishment of human fetal hepatocyte organoids. In Procedure 1, we first describe how to dissect human fetal liver tissue and isolate human fetal hepatocytes. Then, we provide details on how to establish human fetal hepatocyte organoid cultures and maintain them long term. We subsequently describe how to accomplish CRISPR–Cas-based genome engineering of these human fetal hepatocyte organoid cultures (both gene knockin and gene knockout), including details on the transfection procedures, and how to establish clonal lines. Freeze–thaw procedures are described in Box 1. In Procedure 2, we describe how to accomplish CRISPR–Cas-based genome engineering of human adult liver ductal organoid cultures (the derivation and maintenance of these cultures has been detailed elsewhere[31]). We describe the transfection method in depth and the strategy to obtain clonal lines for this organoid system, which differs from the hepatocyte organoids. Finally, in Procedure 3, we first describe how gene knockin can be multiplexed with gene knockout and how multi-gene knockin/knockouts can be achieved in both two liver organoid cultures. We then describe downstream applications of the two genome-engineered organoid cultures, including their genotyping, whole-mount staining and live imaging of fluorescence-based gene knockin lines. Detailed considerations about the experimental design for the different steps of this protocol are detailed below.

Isolation of human fetal hepatocytes and establishment of organoid lines

As discussed in more detail in the 'Limitations' section, the starting point for generation of human fetal hepatocyte organoids is human fetuses. For the isolation of human fetal hepatocytes, fresh material should be processed as soon as possible after recovery of the liver (Fig. 2a). Depending on the gestational age of the donor, white tree-like structures could be evident in the liver parenchyma. In this case, it is recommended that these be removed to avoid contamination of ductal organoid outgrowth. Collagenase type IV digestion in combination with manual mincing is used to isolate the hepatocytes. The appropriate enzymatic activity and the right timing of digestion is crucial to obtain high-quality hepatocytes, upon which successful line establishment is dependent (Fig. 2b,c). Our protocol relies, furthermore, on enrichment of the hepatocytes through steps of low-speed centrifugation. We have not tried to purify hepatocytes by FACS right after isolation. However, given our success in growing hepatocyte organoids after FACS when the line has been established, we envision that sorting of specific subpopulations during isolation will also result in organoid outgrowth. Stromal and non-parenchymal cells might be present in the culture for the first two or three



passages. These cells are normally progressively lost upon passaging. Sometimes we also see a few cystic ductal-like organoids appearing during line establishment, presumably as a consequence of ductal cell outgrowth contaminating the culture (Fig. 2d). If this happens, these cystic organoids should be manually picked and removed from the culture, as their presence impairs the establishment of a pure hepatocyte organoid culture. We consider a hepatocyte organoid line to have been successfully established once passage 5 is reached, as, in our experience, this threshold typically indicates future robust hepatocyte proliferation.

◀ **Fig. 2 | Human fetal hepatocyte organoid line establishment. a**, Schematic of human fetal hepatocyte isolation and seeding into organoid culture. Fresh human fetal liver material (1) is optionally first processed to remove biliary structures, if present, by using scalpel and forceps, as shown in the cartoon in the lower panel. The liver tissue is then chopped using the scalpel and digested by a short collagenase type IV treatment to isolate the hepatocytes (2). The digested tissue is filtered, and low-speed centrifugation is performed to enrich for hepatocytes; removal of RBCs is optional but recommended (3). The cells are mixed with BME and plated into small droplets (4). A representative image of freshly seeded cells is shown in (5). **b**, Representative bright-field images of human hepatocyte outgrowth and organoid formation. Hepatocyte organoids appear after 3–4 d, presenting with a dense morphology. Typically, after 7–10 d of outgrowth, organoids are ready to be split. **c**, Representative bright-field images of different human hepatocyte organoid lines (ranging from passage 7 to 25). Note that variation in hepatocyte organoid shapes within and between lines is expected, presenting typically as spherical or more grape-like structures. The morphology of each line remains constant during prolonged passaging. **d**, Representative bright-field images showing cystic ductal-like organoid outgrowth (black arrowheads) during hepatocyte organoid outgrowth at early passages, which should be manually picked out to obtain pure hepatocyte organoid cultures. Scale bars: **a**, 1,000 μm; **b**, 1,000 μm, high mag, 250 μm; **c**, low mag, 400 μm, and high mag, 250 μm; **d**, 300 μm.

Genome engineering of human fetal hepatocyte and human liver ductal organoids

Genome editing of human fetal hepatocyte and human liver ductal organoids requires two different experimental procedures (Figs. 3 and 4). For human fetal hepatocyte organoids, a cuvette electroporation-based transfection strategy is employed. To this end, organoids have to be dissociated into single cells or small clumps of cells, and, hence, it is recommended to first probe the capacity of the line to grow out after single-cell dissociation. It is recommended to initiate genome engineering on hepatocyte organoid lines from passage 5 and onwards, as these hepatocytes have undergone multiple rounds of proliferation, which normally indicates the robustness of a line. The capacity of the hepatocyte organoid lines to be electroporated does not typically deteriorate with time, and we have successfully performed genome engineering on human fetal hepatocyte organoids at least until passage 50.

In contrast, for human liver ductal organoids, transfection is performed on intact organoids. However, because later steps require a single-cell dissociation step to obtain clonal genome-edited lines, single-cell outgrowth should also be tested before undertaking transfection. Because human liver ductal organoids can be cultured for ~15–20 passages, we recommend initiating genome-editing experiments rapidly after establishing the wild-type line. When starting transfections with organoids at passage 1, clonal genome-engineered human liver ductal organoid lines are normally established by passage 5–6, which allows for many possible downstream applications. We further recommend performing a small pilot transfection experiment before performing the complete experiment to probe the suitability of the line for genome engineering.

Our experience has, thus far, relied on CRISPR–Cas9-mediated genome engineering using the conventional SpCas9 with NGG PAM recognition. In principle, any type of Cas protein capable of introducing a DSB could be used for gene knockout, such as those with widened PAM recognition[21,51,52]. In addition, cytosine or adenine base editors could constitute useful assets to introduce specific base changes for making precision genetic modifications[53,54] as well as to introduce STOP codons[55]. The recent advance of prime editing should further enable and broaden genome-editing possibilities[56]. For gene knockin, because CRISPR-HOT relies on NHEJ instead of HDR, a targeting plasmid containing the exogenous DNA to be knocked-in (e.g., a fluorescent tag) should be used that can be linearized by SpCas9 (in principle, any Cas9 capable of inducing a DSB could be used) through incorporation of a non-human single guide RNA (sgRNA) sequence upstream of the exogenous DNA.

Different strategies to select for genome-engineered organoids might need to be pursued if this protocol is adapted for use with different types of gene editing, as appropriate methods for selection can be dependent on the type of genome engineering and locus targeted. Table 1 summarizes the different gene knockin and knockout strategies that we recommend users of this specific protocol follow.

Limitations

The ability to establish human fetal hepatocyte organoid cultures is limited by accessibility to human fetal material, which can be restricted in some countries owing to ethical and legal restrictions. Appropriate national and institutional regulations must, therefore, be followed and appropriate institutional approvals, and any other required approvals, obtained. It is important that this protocol is carried out only on fetal material obtained following recognized international guidelines with regard to informed consent.

Not all our established human fetal hepatocyte organoid lines have demonstrated clonal outgrowth. As this is essential for genome engineering purposes and potential additional applications that require successful single-cell outgrowth, clonal outgrowth ability should be tested for any line

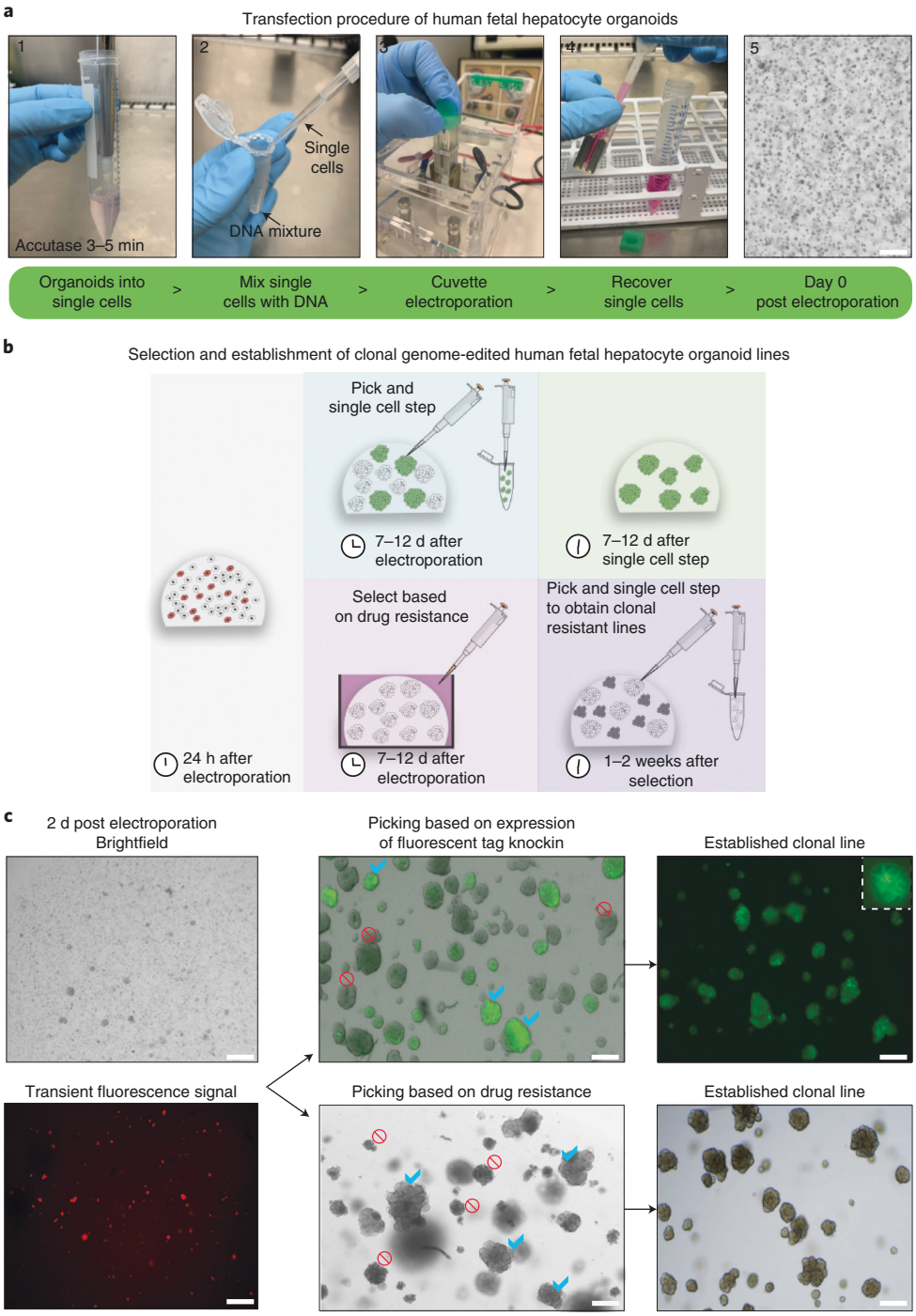


Fig. 3 | Genome engineering of human fetal hepatocyte organoids. a, Schematic of the transfection procedure used. Human hepatocyte organoids are collected and digested into single cells by a short accutase treatment (1). Single cells are mixed with DNA (2) and subsequently electroporated in a cuvette (3). Cells are then recovered (4) and plated in a BME droplet for organoid formation (5). **b**, Schematic representation of the strategies used to establish clonal genome-engineered human fetal hepatocyte organoid lines after electroporation based either on knockin fluorescence (top) or drug selection (bottom). Timing for the single steps is indicated. **c**, Representative bright-field and fluorescent images of hepatocyte organoid outgrowth 2 d after electroporation and typical electroporation efficiency (as assessed by fluorescence; left). Depending on the genome engineering strategy used, outgrowing organoids are picked based on fluorescence expression deriving from the gene knockin, or, otherwise, drug resistance can be applied to enrich for genome-edited organoids. Blue ticks represent fluorescence-positive or drug-resistant organoids to be picked; red symbols represent organoids that have not survived drug selection that should not be picked (middle). Representative images of clonal lines established from a single picked organoid (right). Scale bars: **a**, 1,000 μm; **c**, left, 1,000 μm, and middle and right, 300 μm.

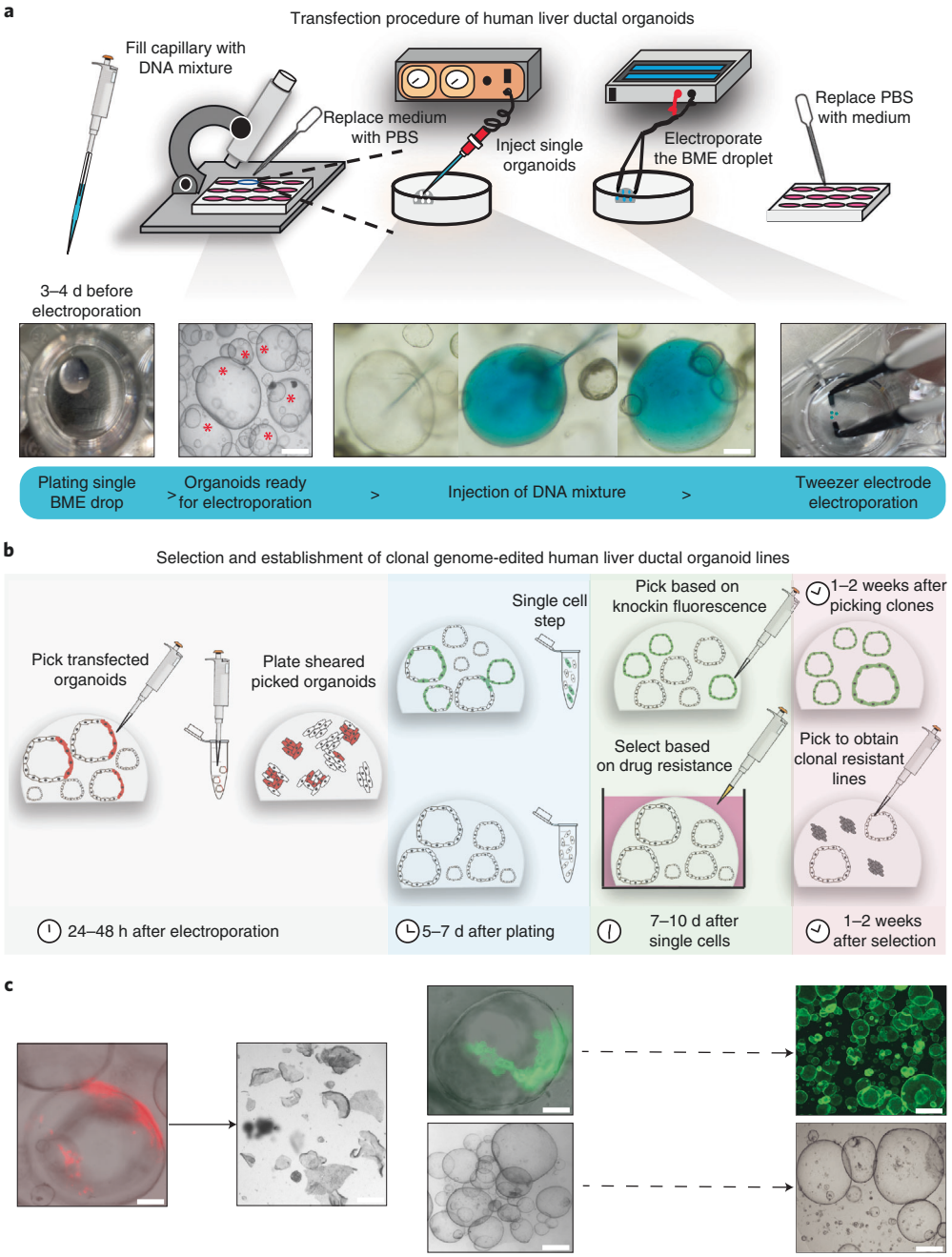


Fig. 4 | Genome engineering of human liver ductal organoids. a, Schematic representation of the transfection procedure used. Human liver ductal organoids are plated into a single BME droplet on the side of a well of a 12-well plate. Organoids are grown for a few days until a proper size for subsequent injection is reached. Before electroporation, the medium in the well is replaced with PBS. The plate is then positioned under the dissection microscope. The capillary is filled with the DNA mixture, mounted on the holder, after which the DNA is injected into the organoids. The BME droplet is then electroporated two or three times using tweezer electrodes. After this procedure, the PBS in the well is replaced with fresh medium. In the bottom panel, pictures of critical details of the procedure are shown, including, from left to right: positioning of the drop in the well before electroporation; correct sizes of the organoids suitable for electroporation (asterisks indicate organoids with the right size); injection of an organoid with the DNA mixture; and position of the electrodes during electroporation **b**, Schematic representation of the strategies used to establish clonal genome-engineered human liver ductal organoid lines after electroporation based either on knockin fluorescence (top) or drug selection (bottom). Timing for the single steps is indicated. **c**, Corresponding to the steps in **b**, representative images of the main steps are shown. From left to right: a composite fluorescent and bright-field image showing a representative example of an electroporated (as judged by fluorescence signal) intact organoid; a bright-field image showing an example of organoids that are picked and sheared into small pieces; a representative example of an organoid showing knocked-in cells that can be picked and dissociated into single cells to establish clonal lines and a representative clonal genome-engineered human liver ductal organoid line (KRT19::mNEON) (top); representative bright-field images of a culture before and after single-cell dissociation and drug selection (bottom). Scale bars: **a**, left, 250 μm, and right, 100 μm; **c**, from left to right: 100 and 50 μm, top: 100 and 300 μm, bottom: 250 and 250 μm.

Table 1 | Gene knockin and knockout strategies

Experimental aim	DNA mixture	Strategy for selection of transfected cells, for both gene knockout and knockin	Strategy for selection of genome-edited organoids
Generation of knockout lines	Plasmid(s) containing the sgRNA specific for the gene of interest[a]	*Enriching for transfected cells in human hepatocyte organoids—optional.* • FACS of transfected cells based on ectopic expression of a fluorescent marker contained in one of the plasmids[b] • Co-transfection with a drug resistance system that can be integrated into the transfected cells, such as the PiggyBac transposase system *Enriching for transfected cells in human liver ductal organoids—mandatory.* Pick and replate the transfected organoids based on ectopic expression of a fluorescent marker contained in one of the plasmids. To further enrich, co-transfection with a drug resistance system that can be integrated into the transfected cells, such as the PiggyBac transposase system, can be used	*Knockout of genes with known selection strategy.* Apply drug selection or withdrawal of medium components and genotype the clonal outgrowing organoid lines *Knockout of genes with unknown selection strategy.* Enrich for transfected cells (recommended) and genotype multiple clonal outgrowing organoid lines
Generation of knockin lines	• Plasmid containing the sgRNA specific for the gene of interest[a] • Self-cleavable targeting plasmid containing the tag of interest preceded by a non-human sgRNA sequence to linearize the plasmid • Plasmid containing the sgRNA to linearize the targeting plasmid[a]		*For tagging of constitutively expressed genes.* Pick the positive organoids based on knockin fluorescence signal and grow and genotype the clonal outgrowing organoid lines *For tagging of non-constitutively expressed genes.* Enrich for transfected cells (recommended) and genotype multiple clonal outgrowing organoid lines

[a]One of these plasmids should contain the Cas9 and a fluorescent marker under control of an independent promoter as a readout of transfection. [b]Efficiency and successful outgrowth after FACS is line dependent.

Table 2 | Efficiencies of line establishment, electroporation and genome engineering of human fetal hepatocyte organoids and human liver ductal organoids

Organoid type	Line establishment efficiency	Proportion of successfully genome editable lines	Electroporation efficiency	Genome engineering efficiency[a,b]	Amount of correctly genome-edited clones obtained
Human fetal hepatocyte organoids	70% (14 out of 20 tested)	70% (5 out of 7 tested)	~9–14%	~5–40% KI, ~5–50% KO	~5–100 clones
Human liver ductal organoids	80% (32 out of 40 tested)	80% (20 out of 25 tested)	~10%	~3–8% KI, ~3–30% KO	~5–25 clones

[a]Within the transfected population. [b]The genome engineering efficiency is highly dependent on the locus targeted and the sgRNA used. We provide a minimum and maximum value based on our experience. KI, knockin; KO, knockout.

that you propose using for such assays. Although most hepatocyte organoid lines are able to regrow after a single-cell dissociation step, for some lines, the regrowth is very slow. In addition, the capacity to tolerate transfection by electroporation differs between lines. We have not observed any correlation with the age and sex of the fetus. Nevertheless, differences in results obtained from the different sexes might affect the results obtained in further specific studies of the organoids; thus, the effect of the sex of the fetus from which material was extracted should be considered.

In our hands, 70% of tested established hepatocyte organoid lines can be robustly genome engineered (Table 2). The inter-donor variability in terms of line establishment, single-cell outgrowth and transfection tolerance can be expected owing to working with primary material. With regard to the

phenotype of the human fetal hepatocyte organoids, expression of the mRNA of some hepatocyte markers is equivalent to those seen in PHH[52] (Supplementary Fig. 1). The fetal origin of the hepatocytes should be considered when deciding whether the downstream application might be appropriate, as drug metabolism and toxicity outcomes could differ between fetal and adult hepatocytes. Hepatocyte organoid cultures from adult human liver can currently only be cultured for few passages[52], and their capacity to be genome edited has not been probed. Future optimization of culture conditions for adult human hepatocyte organoid cultures might support their more robust and long-term expansion and genome engineering.

Human adult liver ductal organoids can be robustly derived from different donors (both healthy and diseased; Table 2) and can be grown from single cells. These organoids are derived from $EpCAM^+$ biliary epithelial cells[14], although their exact origin with regard to the heterogeneity in the cellular composition of the human biliary epithelium is currently uncharacterized. For successful genetic engineering, these organoids must be injected with DNA and electroporated as intact organoids in the BME drop, as described in this protocol. Although this method is more laborious than cuvette electroporation, it ensures outgrow and clonal line establishment of genome-engineered organoids. With this method, the vast majority of human liver ductal organoid lines can be successfully genome edited (Table 2). The expansion time of human liver ductal organoids is normally limited to ~15–20 passages (>6 months), which restricts the period of genetic manipulation and establishment of genome-engineered lines to starting with early-passage organoids (passage 1–3) if extensive downstream analyses are to be performed. Genome-engineered human liver ductal organoid lines can be maintained for at least 3 months in culture with similar proliferation rates (although this might be dependent on the gene targeted). It is recommended to freeze down some of the genome-engineered human liver ductal (as well as the human fetal hepatocyte) organoids to allow ample material for downstream applications.

In principle, there are no restrictions for the generation of knockout lines for both organoid systems, besides the fact that knockout of certain genes could result in organoid lethality. Instead, for the generation of knockin lines, low or absent expression of the endogenous targeted gene, as well as the variable chromatin accessibility of certain loci, can negatively affect successful tagging.

Materials

Reagents

- A83-01 (Tocris Bioscience, cat. no. 2939)
- Accutase (Thermo Fisher Scientific, cat. no. 00-4555-56)
- Advanced DMEM/F12 (Thermo Fisher Scientific, cat. no. 12634010)
- B27 supplement minus vitamin A serum-free, 50× (Thermo Fisher Scientific, cat. no. 12587010)
- BME Type 2, RGF Cultrex Pathclear (BME; R&D Systems, cat. no. 3533-005-02)
- DMSO (Sigma-Aldrich, cat. no. D8418)
- [Leu15]-gastrin I human (gastrin; Sigma-Aldrich, cat. no. G9145)
- GlutaMAX supplement, 100× (Thermo Fisher Scientific, cat. no. 35050-061)
- HEPES, 1 M (Thermo Fisher Scientific, cat. no. 15630-056)
- Hygromycin B Gold Solution (Invivogen, cat. no. ant-hg-1)
- *N*-acetyl-L-cysteine (Sigma-Aldrich, cat. no. A9165)
- Nicotinamide (Sigma-Aldrich, cat. no. N0636)
- Nutlin-3a (Cayman Chem, cat. no. 548472-68-0)
- Penicillin–streptomycin, 10,000 U/ml (Thermo Fisher Scientific, cat. no. 15140-122)
- PBS (Sigma-Aldrich, cat. no. P4417)
- Primocin, 50 mg/ml (Invivogen, cat. no. ant-pm1)
- Recombinant human EGF (hEGF; PeproTech, cat. no. AF-100-15)
- Recombinant human FGF-10 (hFGF-10; PeproTech, cat. no. 100-26)
- Recombinant human HGF (hHGF; PeproTech, cat. no. 100-39)
- Recovery cell freezing medium (Invitrogen, cat. no. 12648-010)
- ROCK inhibitor Y-27632 dihydrochloride (ROCK inhibitor (Y-27632); Abmole Bioscience, cat. no. M1817)
- RSPO1-CM (in-house production; see Broutier et al.[31] for a detailed description on how to make RSPO1-CM and see de Lau et al.[57] and Korinek et al.[58] for how to assess the quality of the RSPO1-CM)

Equipment

- Biosafety cabinet (Telstar, model BioVanguard Green Line)
- Cell culture dish, 100 × 20 mm (Greiner Bio-One, cat. no. 664 160)
- CO_2 cell culture incubator at 37 °C and 5% (vol/vol) CO_2 (Panasonic, cat. no. MCO-19AICUV-PE)
- Conical tube, 15 ml (Greiner Bio-One, cat. no. 188271)
- Conical tube, 50 ml (Greiner Bio-One, cat. no. 227661)
- Cryogenic vial, 1 ml (Greiner Bio-One, cat. no. 123263)
- Freezing container (CoolCell LX, Biocision, cat. no. BCS-405)
- Light microscope (we use a Nikon Eclipse TS100, and this was used to produce images shown in Figs. 1d, 2a–c, 3a,c, 4a,c and 5a–d)
- Liquid nitrogen tank (Taylor Wharton, model K series cryogenic system)
- Microcentrifuge (Eppendorf, model no. 5424)
- Microcentrifuge Safe-Lock tube, 1.5 ml (Eppendorf, cat. no. 0030120086)
- Petri dishes, polystyrene, 100 mm × 20 mm (Sigma-Aldrich, cat. no. P5606)
- Petri dishes, polystrene, 145 mm × 20 mm (Greiner Bio-One, cat. no. 639160)
- Pipette, Pipetman Classic P1000 (Gilson, cat. no. F123602)
- Pipette, Pipetman Classic P20 (Gilson, cat. no. F123600)
- Pipette, Pipetman Classic P200 (Gilson, cat. no. F123601)
- Plugged disposable glass Pasteur pipette, 150 mm (VWR, cat. no. 612-7198)
- Serological pipette, 10 ml (Gosselin SAS, cat. no. GPN10E1)
- Serological pipette, 25 ml (Gosselin SAS, cat. no. GPN25E1)
- Serological pipette, 5 ml (Gosselin SAS, cat. no. GPN5E1)
- Suspension cell culture plate, six-well (Greiner Bio-One, cat. no. 657185)
- Suspension cell culture plate, 12-well (Greiner Bio-One, cat. no. 665102)
- Suspension cell culture plate, 24-well (Greiner Bio-One, cat. no. 662102)
- Suspension cell culture plate, 48-well (Greiner Bio-One, cat. no. 677102)

Reagent setup

A83-01

Dissolve 10 mg of A83-01 in 4.75 ml of DMSO to make a 5 mM stock solution. Avoid freeze–thaw cycles by making small-volume aliquots. Store at −20 °C for up to 2 months.

BME resuspension solution

Prepare a solution of BME and wash buffer in a 3:1 ratio (e.g., mix 600 µl of BME with 200 µl of wash buffer). ▲ **CRITICAL** Do not store; prepare fresh every time.

Gastrin

Dissolve 1 mg of gastrin in 4.8 ml of PBS to make a 100 µM stock solution. Avoid freeze–thaw cycles by making small-volume aliquots. Store at −20 °C for up to 2 months.

hEGF

Dissolve 1 mg of hEGF in 2 ml of PBS with 0.1% (wt/vol) BSA to make a 500-µg/ml stock solution. Avoid freeze–thaw cycles by making small-volume aliquots. Store at −20 °C for up to 2 months.

hFGF-10

Dissolve 100 µg of hFGF-10 in 1 ml of PBS with 0.1% (wt/vol) BSA to make a 100-µg/ml stock solution. Avoid freeze–thaw cycles by making small-volume aliquots. Store at −20 °C for up to 2 months.

hHGF

Dissolve 100 µg of hHGF in 1 ml of PBS with 0.1% (wt/vol) BSA to make a 100-µg/ml stock solution. Avoid freeze–thaw cycles by making small-volume aliquots. Store at −20 °C for up to 2 months.

N-acetyl-L-cysteine

Dissolve 815.95 mg of *N*-acetyl-L-cysteine in 10 ml of water to make a 500-mM stock solution and filter-sterilize the solution. Avoid freeze–thaw cycles by making aliquots. Store at −20 °C for up to 2 months.

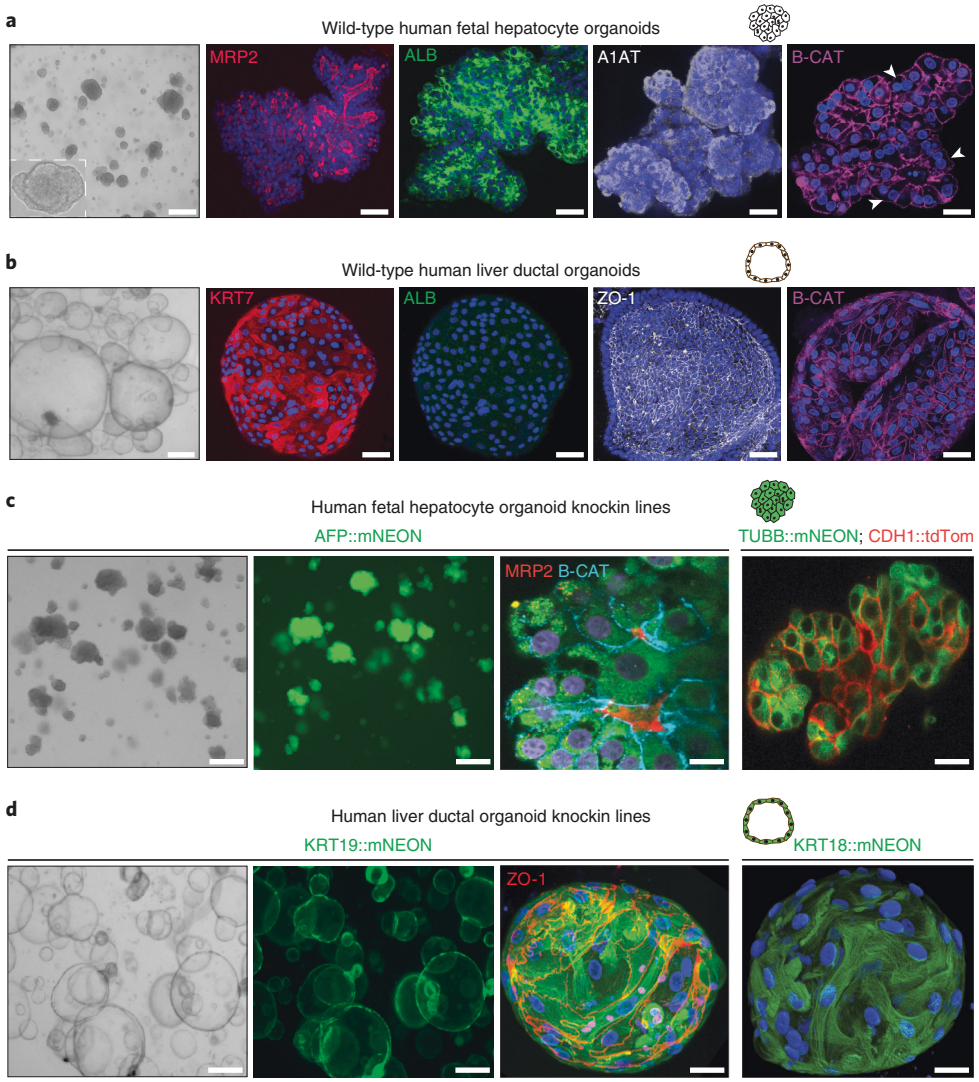


Fig. 5 | Representative follow-up and characterization of human fetal hepatocyte organoids and genome-engineered human liver ductal organoids. **a**, Representative images of bright-field and whole-mount stainings of wild-type human fetal hepatocyte organoids. From left to right: bright-field image of a wild-type human fetal hepatocyte organoid culture line; fluorescent images showing the typical bile canalicular network marked by MRP2; the abundant expression of the hepatocyte markers ALB and A1AT; and characteristic B-CAT staining marking the cell membrane reveals the presence of binucleated hepatocytes within the organoid (white arrowheads). **b**, Representative images of bright-field and whole-mount stainings of wild-type human liver ductal organoids. From left to right: bright-field image of a wild-type human liver ductal organoid culture line; fluorescent images showing the typical expression of the biliary marker KRT7; the absence of expression of the hepatocyte markers ALB; the polarity of ductal organoids as revealed by ZO-1 staining; and characteristic B-CAT staining marking the cell membrane, with no binucleated cells present. **c**, Representative images of genome-engineered human fetal hepatocyte organoids. From left to right: bright-field and fluorescent images showing a clonal AFP::mNEON organoid culture; an AFP::mNEON organoid stained for MRP2 and B-CAT; and a double TUBB-tagged mNEON and CDH1-tagged tdTomato hepatocyte organoid reporter line. **d**, Representative images of genome-engineered human liver ductal organoids. From left to right: bright-field and fluorescent images showing a clonal KRT19::mNEON organoid culture; a KRT19::mNEON organoid stained for ZO-1; and a high-resolution image of a KRT18::mNEON organoid. Organoids in **a** are from passage 21; organoids in **b** are from passage 8. Organoids in **c** and **d** are 5–10 passages after genome engineering. The morphology of the two (genome-engineered) organoid culture systems should present like the images shown throughout all passages. Scale bars: **a**, bright-field image, 400 µm, and fluorescent images, 100 µm; **b**, bright-field image, 200 µm, and fluorescent images, from left to right: 50, 50, 100 and 50 µm; **c**, bright-field image, 300 µm, and fluorescent images, from left to right: 300, 20 and 50 µm; **d**, bright-field image, 200 µm, and fluorescent images, from left to right: 200, 20 and 20 µm.

Nicotinamide

Dissolve 3.05 g of nicotinamide in 25 ml of PBS to make a 1-M stock solution. Avoid freeze–thaw cycles by making aliquots. Store at −20 °C for up to 2 months.

ROCK inhibitor (Y-27632)

Dissolve 50 mg of ROCK inhibitor (Y-27632) in 1.56 ml of sterile dH_2O to make a 100-mM stock solution. Avoid freeze–thaw cycles by making small-volume aliquots. Store at −20 °C for up to 2 months.

Wash buffer

Supplement 500 ml of AdDMEM/F12 medium with 5 ml of 10,000 U/ml penicillin–streptomycin solution, 5 ml of 1 M HEPES and 5 ml of 100× GlutaMAX supplement. Store at 4 °C for up to 2 months.

Design and preparation of sgRNA and targeting plasmid (required for gene knockin by CRISPR-HOT)

The procedure outlined below could, in principle, be used to induce a DSB in the genome with any type of Cas9 protein. We have used SpCas9 to date, which recognizes the conventional NGG PAM sequence. This gene editing approach can, in principle, be performed with any sgRNA; however, the specificity and efficiency of the desired sgRNA and locus accessibility would need to be assessed by the user.

1 Identify the gene of interest for endogenous C-terminus tagging.

2 Design the sgRNA. We recommend identifying potential sgRNAs manually but also screening for potential off-targets. When designing the sgRNA, we recommend that the sgRNA be designed to be as close as possible to the STOP codon to avoid loss of amino acids in the coding sequence of the endogenous gene. It is mandatory that the sgRNA/Cas9 complex mediates the cut either before the STOP codon or in such way that it mediates disruption of the STOP codon. ▲ **CRITICAL** We recommend designing at least 2–3 sgRNAs per genomic locus of interest. If targeting after multiple attempts is still unsuccessful, consider designing sgRNAs in the exon(s) preceding the last exon, as different genomic regions could have different accessibility and, therefore, be more prone to knockin.

3 Clone the designed sgRNAs into the pSPgRNA plasmid according to the protocol described by Ran et al.[59].

4 Choose an appropriate targeting plasmid on the basis of the desired tag. We provided a list of targeting plasmids in Artegiani et al.[23]. Additional targeting plasmids can be easily designed by replacing the existing tags with the desired DNA insert.

5 Choose an appropriate frame selector plasmid to mediate the in-frame insertion of the desired tag, as described in Schmid-Burgk et al.[49]. This is required to linearize the targeting plasmid and increase knockin efficiency via NHEJ. Each frame selector plasmid (0, +1 and +2) linearizes, in an sgRNA/Cas9-mediated manner, the targeting plasmid in a frame-specific manner, leaving a 0-, +1- or +2-nt overhang, through the incorporation of a non-human sgRNA sequence upstream of the tag of interest. To identify the correct frame selector plasmid to use, determine what overhang the sgRNA cut leaves in the open-reading frame of the gene of interest (generally 3 nt upstream of the PAM) and, accordingly, choose the correct frame selector plasmid to mediate expression of the tag of interest in-frame with the gene.

Design and preparation of sgRNA plasmid (required for gene knockout only)

1 Identify the gene of interest to be knocked out using CRISPR–Cas9-mediated NHEJ inactivation.

2 Design sgRNAs to target either the earliest possible exon or, alternatively, the exons containing known functional domains. We recommend identifying potential sgRNAs and screening for potential off-targets using published online tools (e.g., CHOPCHOP[60]). ▲ **CRITICAL** We recommend designing and testing 2–3 sgRNAs per gene of interest.

3 Clone the designed sgRNAs into the pSpCas9-GFP plasmid according to the protocol described by Ran et al.[59]. Alternatively, clone the designed sgRNAs into the pSPgRNA plasmid and use this in combination with a separate plasmid containing the Cas9 and a fluorescent marker under control of an independent promoter as a readout of transfection. ▲ **CRITICAL** If the effect of the introduced gene knockout cannot be easily selected for by drug selection or removal of medium components (for instance, addition of Nutlin-3a can be used to select for functional knockout of *TP53*), we strongly recommend using a transposable drug resistance gene to select for transfected cells by co-transfection of a PiggyBac transposase and drug resistance cassette (e.g., screening based on hygromycin resistance).

Additional materials required for Procedure 1 (Human fetal hepatocyte organoid line establishment and genome engineering)

Reagents

- CHIR-99021 (Sigma-Aldrich, cat. no. SML1046)
- Collagenase from *Clostridium histolyticum*, type IV (Sigma-Aldrich, cat. no. C5138)
- Hoechst 33342 (Life Technologies, cat. no. H3570)

- Human fetal liver **! CAUTION** Ensure that you follow national and local guidelines regarding use of human material and that you obtain appropriate informed patient consent. We recommend that you consult the local medical ethics committee of your institution for advice regarding national regulations and to obtain the necessary permits to collect and process human fetal liver **! CAUTION** We obtained fetal livers from Leiden University Medical Center, The Netherlands. Approval of our study was granted by the medical ethics council of Leiden University Medical Center.
- Opti-MEM reduced serum medium, no phenol red (Life Technologies, cat. no. 11058021)
- Recombinant human KGF (FGF-7) (hFGF-7; PeproTech, cat. no. AF-100-19)
- Recombinant human TGFα (hTGFα; PeproTech, cat. no. 100-16A)
- Sodium chloride 0.9% (wt/vol) (NaCl; Fresenius Kabi)
- Trypan Blue solution, 0.4% (wt/vol), liquid, sterile filtered (Sigma-Aldrich, cat. no. T8154)

Equipment

- Bijou sample container (Sigma-Aldrich, cat. no. Z645346)
- Cell strainer (EasyStrainer), 100 µm (Greiner Bio-One, cat. no. 542000)
- Cuvette chamber (NEPAGENE, cat. no. CU500)
- Cuvette stand holder (NEPAGENE, cat. no. CU600)
- Disposable scalpel, no. 10 (Swann-Morton, cat. no. 0501)
- Fluorescence microscope (we use a EVOS M7000 fluorescence microscope, Thermo Fisher Scientific, and this is used to visualize and/or pick fluorescent transfected or knocked-in organoids)
- Glasstic slide with hemocytometer counting grid (Kova International, cat. no. 87144E)
- Jewelers forceps, Dumont no. 5 (Sigma-Aldrich, cat. no. F6521)
- Nepa electroporation cuvettes, 2-mm gap with pipettes (NEPAGENE, cat. no. EC-002S)
- NEPA21 Super Electroporator (NEPAGENE)
- Round-bottom polystyrene test tube with cell strainer snap cap (35-µm nylon mesh), 5 ml (Corning, cat. no. 352235)
- Whirl-Pak sample bag (sterile container; Sigma-Aldrich, cat. no. WPB01323WA)

Reagent setup

CHIR-99021

Dissolve 1 mg of CHIR-99021 in 715 µl of DMSO to make a 3 mM stock solution. Avoid freeze–thaw cycles by making small-volume aliquots. Store at −20 °C for up to 2 months.

FACS buffer

Dilute DAPI solution 1:750 in wash buffer. ▲ **CRITICAL** Do not store; prepare fresh every time.

HEP digestion buffer

Dissolve 2 mg of collagenase type IV in 20 ml of wash buffer to obtain a final concentration of 100 µg/ml. Pre-heat the HEP digestion buffer in a 37 °C water bath before use. ▲ **CRITICAL** Do not store; prepare fresh every time.

hFGF-7

Dissolve 100 µg of hFGF-7 in 1 ml of PBS with 0.1% (wt/vol) BSA to make a 100-µg/ml stock solution. Avoid freeze–thaw cycles by making small-volume aliquots. Store at −20 °C for up to 2 months.

hTGFα

Dissolve 100 µg of hTGFα in 1 ml of PBS with 0.1% (wt/vol) BSA to make a 100-µg/ml stock solution. Avoid freeze–thaw cycles by making small-volume aliquots. Store at −20 °C for up to 2 months.

Isolation HEP medium

Make up 100 ml of HEP medium and add additional ROCK inhibitor (Y-27632) (100 mM) at a concentration of 5 µl per 10 ml of HEP medium (final concentration of 10 µM). Store at 4 °C for up to 1 month.

Red blood cell lysis buffer

Dilute 10× red blood cell (RBC) lysis buffer in sterile dH_2O to 1×. ▲ **CRITICAL** Do not store; prepare fresh every time.

HEP medium

Use the following volumes to make up 100 ml of medium. Store at 4 °C for up to 1 month.

Component	Volume (to make 100 ml)
Wash buffer	82.04 ml
RSPO-1 CM	15 ml
B27 minus vitamin A	2 ml
Nicotinamide (1 M)	250 µl
N-acetyl-L-cysteine (500 mM)	250 µl
CHIR-99021 (3 mM)	100 µl
Primocin (50 mg/ml)	100 µl
hFGF-7 (100 µg/ml)	50 µl
hFGF-10 (100 µg/ml)	50 µl
hHGF (100 µg/ml)	50 µl
ROCK inhibitor (Y-27632; 100 mM)	50 µl
A83-01 (5 mM)	20 µl
hTGFα (100 µg/ml)	20 µl
hEGF (500 µg/ml)	10 µl
Gastrin (100 µM)	10 µl

Procedure 1 (Human fetal hepatocyte organoid line establishment and genome engineering)

Collection and isolation of human hepatocytes from human fetal liver ● Timing 3–4 h

! CAUTION Consult the local medical ethics committee of your institution for national regulations and to obtain the necessary permits to collect and process human fetal liver.

▲ CRITICAL Before starting hepatocyte isolation, freshly prepare the HEP digestion solution and pre-heat it to 37 °C.

1 Collect human fetal material in sufficient 0.9% (wt/vol) NaCl in a sterile container and transport the material on wet ice to a laboratory with biosafety level II.

■ PAUSE POINT Material can be kept on wet ice for up to 24 h.

2 Remove the excess 0.9% (wt/vol) NaCl by pouring it off and adding fresh 0.9% (wt/vol) NaCl to the material, allow sedimentation of the material and then pour away excess liquid. Repeat until the tissue parts become clearly visible to the naked eye.

3 Depending on the week of gestation (WG), divide the tissue over 2–4 145-mm Petri dishes and add 0.9% (wt/vol) NaCl to each Petri dish and maintain on ice (for example, we usually use two Petri dishes for material from 8–12 WG).

4 Identify the liver under a stereomicroscope with an external light source.

5 Isolate pieces of liver, recognizable by the characteristic coloration, morphology and texture, using laboratory tweezers; transfer to a Petri dish containing 0.9% (wt/vol) NaCl for washing; and transfer to a 7-ml Bijou sample container for storage.

■ PAUSE POINT Tissue can be stored at 4 °C for 24 h. However, directly proceeding to isolation of the hepatocytes is recommended.

6 Transfer the liver tissue to a 15-cm Petri dish.

7 Wash the liver tissue by rinsing it with 10 ml of ice-cold wash buffer.

▲ CRITICAL STEP Check the tissue for presence of white tree-like structures within the liver parenchyma (Fig. 2a). If present, remove these structures as much as possible using tweezers and a scalpel. This step is recommended as it could affect contamination of cystic ductal-like organoid outgrowth during hepatocyte organoid line establishment (Fig. 2d).

8 Aspirate the wash buffer with a glass pipette and pour 5 ml of freshly prepared and pre-heated HEP digestion solution onto the liver tissue.

9 Process the liver tissue by chopping until it is reduced to small pieces using a scalpel and tweezers for 2–5 min (Fig. 2a). Avoid over-digestion of the tissue. As soon as the tissue solution is in predominantly small pieces (~0.2–0.5 mm), move to the next step.

▲ CRITICAL The starting size of the starting material affects the time required for chopping. The size of the starting material is variable because it depends on the age of the donor. For the samples we have processed, it has been between 0.2 and 2 cm, weighing 0.2–2 g.

10 Further homogenize the samples carefully by pipetting with a P1000 pipette.
11 Move the solution to a 50-ml conical-bottom tube.
12 Stop the digestion by adding 35 ml of ice-cold wash buffer.
13 Centrifuge the tube for 5 min at 100*g* at 4 °C.
▲ **CRITICAL** Centrifugation at a low speed is important to enrich for hepatocytes (hepatocytes can be enriched by centrifugation because they are larger than the other cell types in the starting material).
14 Aspirate most of the liquid with a glass pipette until a small volume of liquid containing the cell pellet is left.
▲ **CRITICAL** Ensure that a small amount of liquid is left above the pellet, as the low centrifugation speed results in a loose pellet that is more prone to aspiration.
15 Carefully resuspend the cell pellet in 10 ml of ice-cold wash buffer with a P1000 pipette.
16 Insert a 100-µm filter in a 50-ml conical-bottom tube and pre-wet the filter with 5 ml of wash buffer.
17 Pour the resuspended cell solution from Step 15 through the filter and then add 35 ml of wash buffer to the tube.
18 Centrifuge the tube for 5 min at 100*g* at 4 °C.
19 Aspirate most of the liquid with a glass pipette until a small volume of liquid containing the cell pellet is left. We recommend removal of RBCs at this point, as this is optimal when establishing hepatocyte organoid lines. Proceed with Steps 20–25 to remove the RBCs. Alternatively, proceed directly to Step 26.
20 Carefully resuspend the cell pellet in 2 ml of RBC lysis buffer with a P1000 pipette and transfer the suspension to a 15-ml conical-bottom tube.
21 Incubate for 5 min at room temperature (our usual room temperature is 20–23 °C).
22 Add 3 ml of RBC lysis buffer and mix the solution gently.
23 Centrifuge the tube for 5 min at 400*g* at 4 °C.
24 Wash the cell pellet with 10 ml of ice-cold wash buffer and carefully resuspend the cells with a P1000 pipette.
25 Centrifuge the tube for 5 min at 400*g* at 4 °C.
26 Resuspend the cell pellet in ~500 µl of ice-cold wash buffer and proceed to cell counting.
27 Dilute 10 µl of the cell suspension with 10 µl of Trypan Blue solution and transfer 10 µl to a hemocytometer and count the number of live cells (the ones that do not absorb the dye). In our experience, ~2–5 million cells per gram of fetal liver are obtained using this isolation protocol.
? TROUBLESHOOTING

Seeding of human fetal hepatocytes for organoid formation ● Timing 1–2 h

▲ **CRITICAL** For optimal plating, the suspension culture plates should be pre-heated to allow better setting of the BME. We recommend pre-heating the plates at 37–55 °C for at least 30 min before seeding the cells. In addition, pre-heat the HEP isolation medium in a 37 °C water bath for 30 min before supplying the medium to the cells.
28 Centrifuge the 15-ml tube containing the cells for 5 min at 400*g* at 4 °C.
29 Resuspend the cells in an appropriate volume of BME resuspension solution depending on the number of isolated hepatocytes.
▲ **CRITICAL** For optimal plating of the hepatocytes, 60,000–100,000 cells should be plated in a volume of 100 µl of BME resuspension solution.
▲ **CRITICAL** BME quickly solidifies at room temperature. Therefore, keep the BME on ice when not handling the BME for the entire duration of the seeding experiment.
30 Plate the cells by creating small droplets of BME resuspension solution containing the resuspended cells in a pre-heated suspension culture plate. We recommend using 12-well suspension culture plates and plating 100 µl of the suspension per 12-well plate, making three separate droplets (~33 µl per drop).
31 To allow the BME droplets to solidify, carefully transfer the plate to a cell culture incubator and incubate for ~30 min. This BME concentration is sufficient to prevent cell attachment to the bottom of the plate during gel solidification.
▲ **CRITICAL** Be careful handling the plate containing the BME droplets, as, before they solidify, they are liquid and prone to be disrupted.
32 Upon solidification of the BME droplets, add an appropriate volume of pre-heated HEP isolation medium to the cells. For 12-well plates, add 1 ml; for 24-well plates, add 500 µl.
33 Transfer the plate to a cell culture incubator.

Establishment and culture of human fetal hepatocyte organoid lines ● Timing 6–8 weeks

▲ CRITICAL Successful establishment of hepatocyte organoid lines is donor dependent. We typically obtain success rates ~70% (the success rate being defined as the capacity of a line to reach at least passage 5). We have not observed trends in successful establishment related to the age and sex of the donor.

34 After 2–3 d, small dense organoids should appear in culture (Fig. 2b). When this happens, refresh the medium by aspirating most of the old medium with a glass pipette and supplying the BME droplets with pre-heated HEP medium.
▲ CRITICAL The BME droplets can occasionally detach from the suspension culture plate. Therefore, be careful aspirating the culture medium to avoid loss of loose BME droplets.

35 Replace the culture medium every 2–3 d with new HEP medium. Also, check the size of the organoids every second day and move to the next step when appropriate.

36 When organoids of sufficient size have formed (diameter of 100–250 μm), or when the BME droplet gets crowded, the hepatocyte organoids should be passaged. Organoid passaging can usually be initiated 6–10 d after cell seeding.
▲ CRITICAL Outgrowth/appearance of some non-parenchymal and stromal cells is a normal occurrence at passage 0. These cells are progressively lost upon passaging the hepatocyte organoids. In addition, outgrowth of cystic ductal-like organoids might occur (Fig. 2d). These cystic organoids should be manually picked out from the BME droplet using a P200 pipette under a light microscope. Pure hepatocyte organoid cultures are normally established around passage 2–3.
? TROUBLESHOOTING

37 Aspirate the culture medium with a glass pipette and add an appropriate volume of ice-cold wash buffer to each well (i.e., 1 ml per well of a 12-well plate or 500 μl per well of a 24-well plate).

38 Break the BME droplet containing the organoids in the wash buffer using a P1000 pipette and transfer the mixture to a conical 15-ml tube (three or four wells can be pooled together per 15-ml tube).

39 Use a fire-polished glass pipette to further break the organoids by mechanical shearing into small, but still visible, organoid pieces (diameter of 20–60 μm). Typically, shearing 5–10 times is sufficient.
▲ CRITICAL Be careful not to shear the organoids excessively, as hepatocytes are sensitive cells, and avoid generation of very small pieces of organoids or single cells.

40 Top up the volume in the 15-ml conical tube by adding 10 ml of ice-cold wash buffer.
▲ CRITICAL The cold temperature of the wash buffer is essential to help dissolve the BME.

41 Centrifuge the tube for 5 min at 400*g* at 4 °C.

42 Aspirate the wash buffer and resuspend the organoids in an appropriate volume of BME resuspension solution (100 μl for one well of a 12-well plate in three droplets, 50 μl for one well of a 24-well plate in one droplet).
▲ CRITICAL The optimal splitting ratio for each line should be optimized depending on the specific growth rate of the line. We recommend a regular splitting ratio from 1:2 to 1:5. Particularly when establishing a new line, splitting at a lower ratio (e.g., 1:2 or 1:3) is recommended to probe the growth characteristics of that specific line.
▲ CRITICAL Do not plate the organoids too sparsely, as this could negatively affect line growth.

43 Plate the organoids as described in Steps 30–33. Typically, the hepatocyte organoids have a size of 20–60 μm after shearing.

44 Maintain the line by refreshing with pre-heated HEP medium every 2–3 d and splitting the organoids as described in Steps 36–43. Splitting of the hepatocyte organoids should be performed when the droplets containing the organoids become crowded and when organoids have reached an appropriate size (diameter of 100–250 μm), which is usually every 5–12 d.
▲ CRITICAL It is recommended that organoid cultures be tested for mycoplasma contamination at regular intervals.
? TROUBLESHOOTING

Preparation of the DNA mixture for genome engineering of human fetal hepatocyte organoids ● Timing 30 min–1 h

45 Mix together the required plasmids (see 'Reagent setup' and Table 1) for gene knockin or knockout in a 1.5-ml sterile Safe-Lock tube. A maximum of 20 μg of total DNA resuspended in 20 μl of PBS can be used for each electroporation. The molar ratio of the different plasmids should be considered while preparing the DNA mixture.

Preparation of human fetal hepatocyte organoids for electroporation ● Timing 1–2 h

▲ CRITICAL Use at least two full wells of a 12-well plate (~400,000 cells) for electroporation of human hepatocyte organoids.

▲ CRITICAL Use organoids for electroporation only when they reach a reasonable size (diameter of 100–200 µm) and not immediately after splitting. We recommend using organoids ~4–5 d after splitting.

46 Aspirate the culture medium with a glass pipette and add an appropriate volume of ice-cold wash buffer to each well. An appropriate amount of wash buffer is 1 ml per well of a 12-well plate or 500 µl per well of a 24-well plate.

47 Break the BME droplet containing the organoids in the wash buffer using a P1000 pipette and transfer the mixture to a conical 15-ml tube (3–4 wells can be pooled together per 15-ml tube).

48 Centrifuge the tube for 5 min at 400*g* at 4 °C.

49 Aspirate the wash buffer with a glass pipette.

50 To make the organoids into single cells, add 2 ml of accutase with a P1000 pipette and resuspend the organoid pellet. Incubate the tube for 3–5 min in a 37 °C water bath and occasionally aid single-cell dissociation by manual pipetting the organoids with a P1000 pipette. Inspect whether the organoids are dissociated into single cells (or, alternatively, small clumps) under a bright-field microscope (Fig. 3a).

▲ CRITICAL Avoid excessive dissociation of the organoids. It might be preferable to electroporate small clumps of hepatocytes rather than single cells. This is preferable if the organoid line has limited capacity to grow out from single cells after electroporation. Outgrowth capacity from single cells should ideally be tested before genome engineering of the line.

51 Top up the volume of the tube containing cells with 10 ml of ice-cold wash buffer.

52 Centrifuge the tube for 5 min at 400*g* at 4 °C.

53 Aspirate the wash buffer, wash the cells with 5 ml of ice-cold wash buffer and resuspend the cell pellet carefully with a P1000 pipette.

54 Centrifuge the tube for 5 min at 400*g* at 4 °C.

55 Aspirate the wash buffer and resuspend the cells in an appropriate volume of Opti-MEM. We recommend 130 µl of Opti-MEM per electroporation condition (i.e., to electroporate the cells harvested from two wells of a 12-well plate).

56 Mix the DNA, as prepared in Step 45, together with the cell suspension from the previous step (Fig. 3a).

57 Incubate for 3 min at room temperature.

Electroporation of human fetal hepatocyte organoids ● Timing 1–2 h

58 Transfer the DNA/cell suspension with a P200 pipette into an electroporation cuvette.

59 Electroporate using a NEPAGENE electroporator with the following settings: Poring Pulse (Voltage = 175 V, Pulse Length = 7.5 ms, Pulse Interval = 50 ms, Number of Pulses = 2, Decay Rate: 10%, Polarity: +) and Transfer Pulse (Voltage = 20 V, Pulse Length = 50 ms, Pulse Interval = 50 ms, Number of Pulses = 5, Decay Rate: 40%, Polarity: +/−; Fig. 3a).

60 Immediately after electroporation, add 750 µl of HEP isolation medium to the cuvette. HEP isolation medium is recommended owing to the higher concentration of ROCK inhibitor Y-27632, which is crucial to prevent anoikis of single cells (Fig. 3a).

61 Allow the electroporated cells, still within the cuvette, to recover for 20 min at room temperature. In the meantime, proceed with the next step.

62 Prepare a 15-ml conical tube filled with 8 ml of ice-cold wash buffer.

63 Transfer the cells from the cuvette into the pre-filled 15-ml conical tube, using the small plastic pipette provided with the cuvette (Fig. 3a).

▲ CRITICAL Handle the cells very carefully because cells are in a stressed state after electroporation.

64 Centrifuge the tube for 5 min at 400*g* at 4 °C.

65 Plate the single cells as described in Steps 30–33. We recommend plating the cells at a dense concentration (for example, if two wells of a 12-well plate were used for one electroporation, we recommend plating them in 2–3 wells of a 12-well plate). Alternatively, cells can be plated into 24-well plates to facilitate selection (Fig. 3a).

Establishment of genome-engineered human hepatocyte organoid lines ● Timing 6–8 weeks

66 Twenty-four hours after electroporation, check the health of the cells. If cells were transfected with a plasmid containing a fluorescent protein under the control of an independent promoter, check for

a positive fluorescence signal. For example, if pSpCas9-GFP was used for gene knockout, check for GFP fluorescence. For gene knockin, check for mCherry fluorescence derived from the frame selector plasmid (Fig. 3c). This transient fluorescence signal is expected to be visible for 5–8 d. After successful electroporation, 9–14% of the hepatocytes should be electroporated[23].

? TROUBLESHOOTING

67 Three to five days after electroporation, assess the cells to monitor their growth. At this point, small clumps of cells should be visible. When small clumps of cells are visible, switch to regular HEP medium. At this stage, the hepatocytes can, optionally, be sorted by FACS to select for transfected cells. This step helps to enrich any genome-engineered hepatocytes. However, successful outgrowth after sorting is strongly line dependent, so if the line that you have transfected does not outgrow successfully after single-cell sorting, it is recommended to proceed directly to Step 78 for drug selection or Step 80 for picking. If targeting of a constitutively expressed gene via knockin has been attempted, the fluorescence signal resulting from successful knockin should appear during a relatively short timeframe (3–7 d after electroporation); however, the exact time span over which there is a fluorescence signal can vary depending on the specific targeted gene (Fig. 3c).

? TROUBLESHOOTING

(Optional) FACS of single cells ● Timing 4 h

▲CRITICAL Recommended only for lines able to outgrow after single-cell sorting.

▲CRITICAL Perform FACS 3–5 d after electroporation when the transient fluorescence signal is abundant.

68 To sort single cells from the human hepatocyte organoids by FACS, dissociate the organoids into single cells as described in Steps 46–54. We recommend using half of the cells that were electroporated for FACS.

69 Aspirate the wash buffer and resuspend the cells in 2 ml of ice-cold wash buffer.

70 Filter the cells over a 5-ml FACS tube using a cell strainer snap cap.

71 Centrifuge the tube for 5 min at 400*g* at 4 °C.

72 Carefully aspirate the wash buffer and resuspend the cells in 500 µl of FACS buffer. Mix the cells well.

73 Sort the cells using an FACS sorter.

▲CRITICAL Optimal conditions for FACS need to be determined by the user, as they are dependent on the FACS sorter used.

74 Collect the cells into a 1.5-ml Safe-Lock tube pre-filled with 500 µl of wash buffer.

75 Centrifuge the tube for 5 min at 400*g* at 4 °C.

76 Plate the single cells in one 50-µl BME droplet in a single well of a 24-well plate as described in Steps 30–33.

77 For the first few days after sorting, culture the single cells in HEP isolation medium. Once small organoids start to appear, switch to and maintain the organoids in regular HEP medium.

? TROUBLESHOOTING

(Optional) Drug selection ● Timing variable

▲CRITICAL Any type of selection (including growth factor withdrawal or drug administration) can be applied when the organoids have formed a reasonable size (clumps of ~20–50 cells), which is typically reached from 7–12 d after electroporation onward. This section describes drug selection; for different strategies of selection (Fig. 3b,c), refer to Table 1.

78 Replace medium with the appropriate selection medium, using HEP medium as the base. For example, organoids with *TP53* mutations can be selected for by adding 10 µM Nutlin-3a; Hygromycin B resistance can be selected for by inclusion of 100 ng/ml of Hygromycin B. For any type of drug selection, it is important to also include a negative control (i.e., a well of untransfected cells from the same organoid line that have undergone the same steps as the transfected cells), to serve as the baseline for selection resistance.

79 Maintain drug selection until the effects of the selection are clearly visible (Fig. 3b,c) and control organoids have completely died. Although this timeframe can vary between donors, typically Nutlin-3a selection takes 2 weeks and requires one passaging step. Hygromycin B selection takes ~7–10 d, usually without any need to passage organoids during selection.

Picking of organoids ● Timing 2–4 h

▲CRITICAL Single surviving organoids after selection (from Step 79) or positively knocked-in single organoids (from Step 67 or 77) have to be picked. The optimal size for picking is a diameter of >100 µm

(Fig. 3b). Thus, the optimal time to pick organoids depends on the growth speed of the outgrowing positive organoids; however, it is typically 10–20 d after electroporation.

80 Before organoid picking, prepare 1.5-ml Safe-Lock tubes filled with 150 µl of accutase and maintain them on ice.

81 Using a P200 pipette, slowly insert the tip in the BME droplet, gently aspirate the single desired organoid and transfer immediately into the tube containing accutase. If undertaking gene knockin, several fluorescence-positive organoids can be combined together to generate a bulk culture. Bulk cultures generally have better outgrowth and, as a consequence of starting with more material, allow for quicker expansion of the line. To do this, pick several organoids and place them in the same tube containing accutase.

▲ **CRITICAL** This procedure needs to be performed under a bright-field or a fluorescence microscope to be able to visualize the drug-resistant or fluorescence-positive organoids, respectively.

82 Incubate the tube for 2–3 min in a 37 °C water bath and occasionally aid single-cell dissociation by manually pipetting the organoids with a P200 pipette.

▲ **CRITICAL** Avoid excessive single-cell dissociation as this will negatively affect clonal outgrowth.

83 Top up the tube with 1 ml of ice-cold wash buffer.

84 Centrifuge the tube for 5 min at 400*g* at 4 °C.

85 Aspirate the wash buffer carefully with a glass pipette or, alternatively, use a P1000 pipette, leaving a small volume of wash buffer containing the cell pellet.

86 Wash the cells with 1 ml of ice-cold wash buffer and carefully resuspend the cell pellet.

87 Centrifuge the tube for 5 min at 400*g* at 4 °C.

88 Aspirate the wash buffer carefully with a glass pipette or, alternatively, use a P1000 pipette, leaving a small volume of wash buffer containing the cell pellet.

89 Plate the cells as described in Steps 30–33 in a single well of a 24-well plate or a 48-well plate. For the first few days, culture the organoids in HEP isolation medium. Once small organoids start to appear, switch to regular HEP medium.

▲ **CRITICAL** Clonal outgrowth success rates depend on the hepatocyte organoid line. Typically, 60% of picked single organoids can be expected to grow out.

? TROUBLESHOOTING

90 Passage the organoids (as described in Steps 36–43) when organoids of sufficient size have formed (diameter of 100–250 µm) or when the BME droplet gets crowded. After this passage (the first passage of the (clonal) organoid lines), one well should be used to genotype the line (see Steps 1–16 of Procedure 3). When the genotype is confirmed, if you want to proceed to multiplex gene knockin with gene knockout or generate multi/sequential gene knockin/knockout organoid lines, proceed to Steps 17 and 18 of Procedure 3. Otherwise, proceed to Step 19 of Procedure 3 and consider whether you want to carry out additional experiments (Fig. 1d).

Additional materials required for Procedure 2 (Genome engineering of human liver ductal organoids)

Reagents

- FastGreen (Sigma-Aldrich, cat. no. F7258)
- Forskolin (R&D Systems, cat. no. 1099)
- Human adult liver biopsies

! CAUTION Ensure that you follow national and local guidelines regarding use of human material and that you obtain appropriate informed patient consent.

! CAUTION We obtained adult liver biopsies from Erasmus Medical Center Rotterdam, The Netherlands. Approval of our study was granted by the medical ethics council of Erasmus Medical Center Rotterdam.

- Opti-MEM reduced serum medium, no phenol red (Life Technologies, cat. no. 11058021)

Equipment

- Borosilicate capillaries with filament (Sutter Instruments, cat. no. BF120-69-10)
- External light source (Schott, cat. no. KL 1600 LED)
- Fluorescence microscope (we use an EVOS M7000 fluorescence microscope, Thermo Fisher Scientific, and this is used to visualize and/or pick fluorescent transfected or knocked-in organoids)
- Jewelers forceps, Dumont no. 5 (Sigma-Aldrich, cat. no. F6521)
- NEPA21 Super Electroporator (NEPAGENE)
- P-97 Flaming/Brown pipette puller (Sutter Instruments)

- Pico Pump (WPI, cat. no. PV820)
  ▲ CRITICAL This equipment needs to have a foot switch.
- Stereomicroscope (Leica MZ6, Leica)
- Tweezers with variable gap 2 round platinum plate electrode, 1-mm diameter (NEPAGENE, cat. no. CUY650P1)

Reagent setup

Forskolin

Dissolve 10 mg of Forskolin in 2.44 ml of DMSO to make a 10 mM stock solution. Avoid freeze–thaw cycles by making small-volume aliquots. Store at −20 °C for up to 2 months.

Injection dye

Dissolve 1 mg of FastGreen in 2 ml of PBS to a final concentration of 0.05% (wt/vol). Centrifuge the solution at maximum speed for 2 min at room temperature to get rid of potential aggregates. Store at room temperature for up to 1 year.

DUC medium

Use the following volumes to make up 100 ml of medium. Store at 4 °C for up to 1 month.

Component	Volume (to make 100 ml)
Wash buffer	86.31 ml
RSPO-1 CM	10 ml
B27 minus vitamin A	2 ml
Nicotinamide (1 M)	1 ml
N-acetyl-L-cysteine (500 mM)	250 µl
Primocin (50 mg/ml)	100 µl
Forskolin (10 mM)	100 µl
A83-01 (5 mM)	100 µl
hFGF-10 (100 µg/ml)	100 µl
hHGF (100 µg/ml)	25 µl
hEGF (500 µg/ml)	10 µl
Gastrin (100 µM)	10 µl

Establishment of human liver ductal organoid lines

Establish and culture human liver ductal organoid lines from adult human liver as described in the primary research paper by Huch et al.[14] and the associated protocol by Broutier et al.[31]. The composition of DUC medium is described above.

Procedure 2 (Genome engineering of human liver ductal organoids)

Preparation of human liver ductal organoids for electroporation ● Timing 3–4 d

1 Three to four days before electroporation, split the organoids at a ratio of between 1:4 and 1:6 (as described in refs. [14,31]) in a single 50-µl droplet of BME in a well of a 12-well plate. Do not place the droplet in the center of the well; instead, place it to the side, as this will facilitate easier DNA injection with the capillary.
▲ CRITICAL It is important that organoids reach a sufficient size to be suitable for subsequent injection with DNA and electroporation. Organoids with a diameter of 150–500 µm should be used for the subsequent steps. Organoids with this diameter are typically reached 3–4 d after splitting (Fig. 4a). We recommend performing electroporation on three confluent wells per condition (~200,000 cells).
▲ CRITICAL It is essential that the cells be plated at the correct concentration to allow sufficient growth of the organoids, as this facilitates the DNA injection.

Preparation of the DNA mixture ● Timing 30 min–1 h

2 Mix together the required plasmids (as described in 'Reagent setup' and Table 1) for gene knockin or knockout in a 1.5-ml sterile Safe-Lock tube. We recommend using at least 100 µg of total DNA

resuspended in ~40 µl PBS and the molar ratio of the different plasmids should be considered while preparing the DNA mixture. A plasmid concentration of 3 µg/µl is optimal, but it should be at least 1 µg/µl, as injection of the diluted DNA into the organoid lumen otherwise requires too large an injection volume.

▲ **CRITICAL** It is essential to use a mixture of DNA that contains a plasmid with a fluorescent protein under the control of an independent promoter to allow for picking of successfully transfected organoids based on fluorescence signal.

3 Add the injection dye to the DNA mixture at a concentration of 1 µl per 20 µl of DNA.

Preparation and loading of the capillaries for electroporation of human liver ductal organoids ● Timing 30 min–1 h

4 Prepare glass borosilicate capillaries with a glass puller using the following conditions: Pull = 200, Velocity = 140, Time = 400 for pulling. Because each different electroporation condition requires at least one different capillary, pull enough capillaries for the desired experimental settings.

5 Load one capillary with the DNA mixture using a P20 pipette and a long pipette tip by aspirating 20 µl of the DNA mixture using a long pipette tip, inserting this tip into the capillary open end and filling the capillary with the DNA mixture, while slowly retracting the long pipette tip.

6 Mount the DNA-filled capillary on the nozzle of the capillary holder connected to the pressure pump (Pico Pump).

▲ **CRITICAL** It is important that the pressure pump has a foot switch, because it enables injection of the DNA mixture into the organoids with a foot command while leaving the hands free to hold the plate and inject the organoids with the DNA-filled capillary.

7 Carefully break the extremity of the capillary tip using jewelers forceps, as the tip of the capillary is usually fused at the end.

Electroporation of human liver ductal organoids ● Timing 2–4 h

! **CAUTION** The steps in this section are undertaken in a non-sterile environment outside a tissue culture hood. The plate containing the organoids should only be opened for the necessary steps, and it is important to work in a clean (ethanol-wiped) area.

8 Remove the culture medium from the well of the plate to be electroporated (established in Step 1) with a P1000 pipette and add 2 ml of sterile PBS.

9 Put the plate under an illuminated stereomicroscope. The stereomicroscope is required to visualize the organoids while performing the injection.

10 While holding the plate in one hand and looking at the organoids through the microscope, use the DNA-filled capillary mounted on the capillary holder with the other hand and carefully inject single organoids of sufficient size (optimal diameter 150–500 µm; Fig. 4a). As soon as the capillary is inside the organoid, press the foot pump switch (for ~1–5 s using an eject pressure of 5–10 p.s.i. when using a Pico Pump, but optimal settings should be determined each time because the pressure needed is influenced by the opening of the capillary) to slowly inject the organoids with the DNA mixture until the organoid is fully loaded as visualized by complete dispersion of the injection dye. After the organoid is filled, retract the capillary and proceed to inject the next organoid. To maximize efficiency, inject as many organoids as possible in the same droplet.

▲ **CRITICAL** Injection should be performed very gently to avoid collapse of the organoids.

11 Once sufficient organoids have been injected, use the tweezer electrodes (tip pre-wetted in PBS) to electroporate the BME droplet containing the injected organoids two or three times at different angles around the droplet using a NEPAGENE electroporator by gently squeezing the droplet (Fig. 4a). Electroporation settings as follows: Poring Pulse (Voltage = 50 V, Pulse Length = 10 ms, Pulse Interval = 50 ms, Number of Pulses = 4, Decay Rate: 10%, Polarity: +) and Transfer Pulse (Voltage = 20 V, Pulse Length = 50 ms, Pulse Interval = 50 ms, Number of Pulses = 5, Decay Rate: 40%, Polarity: +/−).

▲ **CRITICAL** During the injection steps, it is important to keep the tip of the tweezer electrodes in PBS to keep them wet.

12 Remove the PBS from the electroporated well and add 2 ml of DUC medium. Proceed to the next well and repeat Steps 10–12. Once all the droplets have been electroporated, transfer the plate to a cell culture incubator.

13 Wash the tweezer electrodes with sterile dH_2O, and dry them before storing.

Establishment of genome-engineered human liver ductal organoid lines ● Timing 6–8 weeks

14 One to two days after electroporation, monitor successful transfection by assessment of the transient fluorescence signal using a fluorescence microscope. Usually the electroporated cells lie on the outside of the organoid and constitute ~10% of the cells within the organoid (Fig. 4c).
? TROUBLESHOOTING

15 Pick all transfected (fluorescence-positive) organoids with a P200 pipette under a fluorescence microscope, as similarly described for human hepatocyte organoids in Step 81 of Procedure 1 and transfer them into a 1.5-ml Safe-Lock tube filled with 500 µl of ice-cold wash buffer.

16 Disrupt the organoids by repeatedly pipetting with a P200 pipette.

17 Centrifuge the tube for 5 min at 400*g* at 4 °C.

18 Resuspend the sheared organoid pieces in BME resuspension solution (volume depends on how many organoids have been successfully transfected and picked) and plate single 50-µl droplets in a well of a pre-heated 24-well suspension culture plate (Fig. 4c). Upon solidification of the BME droplets, add 1 ml of DUC medium.
▲CRITICAL This step is important to enrich for transfected cells.

19 Monitor the growth of organoids. Usually 5–7 d after plating, the organoids will have regrown as regular round cysts, and many electroporated cells should be visible by fluorescence. If knockin lines have been generated, and a constitutively expressed gene has been targeted, the fluorescence signal resulting from successful knockin usually appears 3–7 d after electroporation; however, this can be dependent on the specific targeted gene.
▲CRITICAL Only after this step can you proceed to single-cell digestion by accutase to obtain clonal human liver ductal organoid lines (Fig. 4b). We do not proceed to single-cell digestion immediately after electroporation because this results in very limited outgrowth.
? TROUBLESHOOTING

20 Remove the culture medium and add 1 ml of ice-cold wash buffer to the well with a P1000 pipette. Collect the organoids and transfer the organoids into a 1.5-ml Safe-Lock tube.

21 Centrifuge the tube for 5 min at 400*g* at 4 °C.

22 Aspirate the wash buffer carefully with a glass pipette or, alternatively, with a P1000 pipette, leaving a small volume of wash buffer containing the organoid pellet.

23 Add an appropriate volume of accutase (e.g., 250 µl) to the organoids. Incubate the organoids for 2–3 min in a 37 °C water bath and occasionally aid single-cell dissociation by manual pipetting the organoids with a P200 pipette.

24 Add 1 ml of ice-cold wash buffer to the tube.

25 Centrifuge the tube for 5 min at 400*g* at 4 °C.

26 Aspirate the wash buffer carefully with a glass pipette or, alternatively, with a P1000 pipette, leaving a small volume of wash buffer containing the cell pellet.

27 Wash the cells by adding 1 ml of ice-cold wash buffer to the tube.

28 Centrifuge the tube for 5 min at 400*g* at 4 °C.

29 Resuspend the cell pellet with an appropriate volume of BME resuspension solution and plate a single 50-µl BME droplet per well of 24-well suspension culture plate.

30 After solidification of the BME droplets, add 500 µl of DUC medium supplemented with 10 µM ROCK inhibitor (Y-27632). We recommend supplementation with the ROCK inhibitor (Y-27632) to prevent anoikis of single cells. Typically, ~60% of single cells are able to grow out.

31 Monitor growth of organoids. Seven to ten days after plating, organoids should have regrown from the single cells, appearing as regular round cysts.

(Optional) Drug selection ● Timing variable

▲CRITICAL Any type of selection (including growth factor withdrawal or drug administration; as examples, TP53 mutations can be screened with 10 µM Nutlin-3a, and Hygromycin B resistance can be screened with 100 ng/ml Hygromycin B) can now be applied. This section describes drug selection; for different strategies of selection (Fig. 4b), refer to Table 1.

32 Replace medium with the appropriate selection medium, using DUC medium as the base. For example, organoids with *TP53* mutations can selected for by adding 10 µM Nutlin-3a; Hygromycin B resistance can be selected for by inclusion of 100 ng/ml Hygromycin B. (See Fig. 4b,c and Table 1 for different selection strategies dependent on the experimental setting.) For any type of drug selection, it is important to include a proper control (i.e., a well of untransfected cells from the same

organoid line that have undergone the same steps as the transfected cells), which will serve as the baseline for selection resistance.

33 Maintain drug selection until selection is clearly visible and control organoids have completely died. Although this timeframe can vary between donors, typically Nutlin-3a selection takes 2 weeks and requires one passaging step, and Hygromycin B takes ~7–10 d without the need of passaging (Fig. 4b,c).

Picking of organoids ● Timing 3–5 h

34 Single surviving organoids after selection (from Step 33) or knocked-in single organoids (as visualized by positive fluorescence signal) (from Step 31) have to be picked (optimal size for picking: diameter of >100 µm) to obtain clonal organoid lines (Fig. 4b). For picking, use a P200 pipette under either a bright-field or a fluorescence microscope and transfer immediately the picked organoid into a 1.5-ml Safe-Lock tube filled with 300 µl of ice-cold wash buffer.

35 Disrupt the organoids by repeatedly pipetting with a P200 pipette.

36 Centrifuge the tube for 5 min at 400*g* at 4 °C.

37 Resuspend the sheared organoids in BME resuspension solution and plate a single 25-µl droplet per well of a pre-heated 48-well suspension culture plate. Upon solidification of the BME droplets, add 250 µl of DUC medium. About 60% of the picked clones can regrow into lines.

? TROUBLESHOOTING

38 After the first split, spare a well for genotyping as described in Steps 1–16 of Procedure 3. The clonal genome-engineered organoid lines can then be used for further downstream applications as described in Procedure 3 and Fig. 1.

Additional materials required for Procedure 3 (Genotyping, further genome engineering and downstream applications)

Reagents

- Anti-A1AT rabbit polyclonal 1:500 (Abcam, cat. no. ab9373, RRID: AB_307219)
- Anti-AFP rabbit polyclonal 1:250 (Thermo Fisher Scientific, cat. no. PAS-16658, RRID: AB_10979157)
- Anti-Albumin goat polyclonal 1:500 (Bethyl, cat. no. A80-229A, RRID: AB_67018)
- Anti-B-CAT rabbit polyclonal 1:500 (Santa Cruz Biotechnology, cat. no. sc-7199, RRID: AB_634603)
- Anti-KRT7 mouse monoclonal 1:200 (Millipore, cat. no. MAB3226, RRID: AB_2134453)
- Anti-MRP2 mouse monoclonal 1:100 (Abcam, cat. no. ab3373, RRID: AB_303751)
- Anti-ZO-1 rabbit polyclonal 1:200 (Life Technology, cat. no. 402200, RRID: AB_2533456)
- BSA (Sigma-Aldrich, cat. no. 10-735-094-001)
- DAPI solution, 1 mg/ml (Thermo Fisher Scientific, cat. no. 62248)
- Sodium azide, 0.1 M solution (Sigma-Aldrich, cat. no. 08591-1ML-F)
- Triton X-100 (Sigma-Aldrich, cat. no. X100)

Equipment

- Confocal microscope equipped with a cell culture chamber (we use an Sp8 confocal (Leica), and this was used to produce the images shown in Figs. 1d and 5a–d)
- Glass-bottom plate, 12-well (Cellvis, cat. no. P12-1.5H-N)
- Sensoplate, 24-well (Greiner Bio-One, cat. no. 662892)
- Sensoplate, 96-well (Greiner Bio-One, cat. no. 655892)

Reagent setup

Blocking buffer

Dissolve 500 mg of BSA in 10 ml of PBS to a final BSA concentration of 5% (wt/vol). Filter the solution with a 0.45-µm filter. Prepare fresh and store at 4 °C for 1 week.

DAPI-containing staining wash buffer

Dilute the DAPI solution (1 mg/ml) 1:1,000 in staining wash buffer to a final concentration of 1 µg/ml. Do not store; prepare fresh every time.

Lysis buffer

Combine 0.1M Tris-HCl (pH 8.5–9.0), 0.2 M NaCl, 5 mM EDTA and 0.2% (wt/vol) SDS in sterile dH_2O. Store at room temperature for up to 2 years.

Permeabilization buffer
Dilute the Triton X-100 stock solution (20%) 1:40 in PBS to a final Triton X-100 concentration of 0.05% (vol/vol). Do not store; prepare fresh every time.

Preservation staining buffer
First prepare sodium azide–PBS solution by adding 1 ml of 0.1 M sodium azide solution to 1 L of PBS. This solution can be stored at room temperature for up to 1 year. Use the sodium azide–PBS solution to dissolve 100 mg of BSA in 10 ml of PBS to a final BSA concentration of 1% (wt/vol). Filter the preservation staining buffer with a 0.45-μm filter. Prepare fresh and store at 4 °C for 1 week.

Staining wash buffer
Dissolve 100 mg of BSA in 10 ml of PBS to a final BSA concentration of 1% (wt/vol). Filter the solution with a 0.45-μm filter. Prepare fresh and store at 4 °C for up to 1 week.

Triton X-100 stock solution (20%)
Dissolve 4 ml of Triton X-100 in 16 ml of H_2O to a final Triton X-100 concentration of 20% (vol/vol). Heat up the solution until dissolved; it might take a while to be completely dissolved. Store at room temperature for up to 1 year.

Genotyping primers
Before starting genotyping of the organoid lines, genotyping primers should be designed for the potential targeted genomic region of interest. In case of gene knockin, primers should span the region of insertion, to be able to check for correct insertion, amplifying a product containing the region of sgRNA targeting fused to the piece of inserted DNA. In case of gene knockout, the region of PCR amplification should flank the expected region of sgRNA targeting. In general, it is recommended to design primers in such way that PCR products of 400–800 bp are generated. We recommend designing at least two sets of genotyping primers to ensure amplification of the right genomic region.

Procedure 3 (Genotyping, further genome engineering and downstream applications)

Genotyping of genome-engineered organoids from human liver ● Timing 3–5 d

▲ **CRITICAL** Use the spare well containing clonal lines from the genome-engineered organoids for genotyping.

1 Aspirate the culture medium and harvest the BME droplet containing the organoids in 1 ml of ice-cold wash buffer using a P1000 and transfer the suspension to a 1.5-ml Safe-Lock tube.
2 Centrifuge the tube for 5 min at 400*g* at 4 °C.
3 Discard the wash buffer and resuspend the organoid pellet in 400 μl of lysis buffer.
4 Incubate the tube for 2–3 h at 55 °C.
5 Add 500 μl of isopropanol to the tube and mix the well by shaking.
6 Centrifuge the tube for 15 min at full speed (>12,000*g*) at room temperature.
7 Inspect the tube for the presence of a white pellet that contains the DNA. Remove the supernatant and wash the pellet with 1 ml of 70% (vol/vol) EtOH.
8 Centrifuge the tube for 5 min at full speed (>12,000*g*) at room temperature.
9 Remove the supernatant and allow the pellet to dry.
10 Depending on the pellet size, add an appropriate volume of dH_2O and resuspend the pellet. Typically, 20–50 μl of dH_2O is sufficient to allow complete resuspension of the DNA.
11 Measure the DNA concentration and quality.
■ **PAUSE POINT** DNA can be stored at −20 °C until further use.
12 Perform a PCR reaction on the genomic DNA with the designed genotyping primers using appropriate standard protocol. Usually, an input of 2 ng of genomic DNA suffices.
13 Run the PCR product on a 1% (wt/vol) agarose gel and extract the PCR product from the gel.
14 Sanger sequence the PCR product and analyze the results.
15 If you have undertaken gene knockout, subclone the PCR product to assess the genomic modifications on each single allele. This is required because the two alleles are likely modified in different ways. If you have undertaken gene knockin, subcloning of the PCR product is not required because, in the vast majority of cases, the exogenous DNA is incorporated in a heterozygous manner.
16 If you confirmed by genotyping that a knockout organoid line has been generated, we highly recommend confirming functional gene knockout at the protein level by western blotting.

Multiplexing gene knockin with gene knockout or generation of multi/sequential gene knockin/knockout organoid lines ● Timing 6–8 weeks

▲ CRITICAL For the generation of double or multiple knockin lines using CRISPR-HOT, the tagging of endogenous genes has to be performed sequentially. This is mandatory, because insertion of the desired tag at the right locus is uncontrollable, and the likelihood of simultaneous insertion of each tag correctly at each different locus is very low. In addition, different tags should be used to tag different genes. To generate a combined gene knockin and gene knockout organoid line, it is mandatory that these steps are performed sequentially because the exogenous DNA could insert at the desired locus for gene knockout, which is an unwanted outcome. In most cases, gene knockin should be performed before gene knockout if gene function is to be studied. For the generation of double/multiple gene knockout lines, two options can be considered. First, multiple sgRNAs can be co-electroporated together within one experiment to generate multi-gene knockout organoids within one experiment. Alternatively, if mutations are to be introduced in a sequential order, another/multiple round(s) of gene knockout can be performed on an established knockout organoid line.

17 Once the first knockin or knockout organoid line has been generated and established, it should be expanded until enough material can be spared for a subsequent round of genome engineering (multiple gene knockin or gene knockout or a combination of the two). We recommend using two wells of a 12-well plate for hepatocyte organoids and ~4–6 wells of a 24-well plate for human liver ductal organoids as starting material for the electroporation.

18 Perform genome engineering for the respective organoid system and genotyping as described in the appropriate earlier section.

Downstream applications of genome-engineered organoids from human liver ● Timing is variable depending on the application

19 Genome-engineered organoid lines can be whole-mount stained, as described in option A for human fetal hepatocyte organoids and in option B for human liver ductal organoids. Alternatively, organoids can be live imaged as described in option C.

(A) **Whole-mount staining of genome-engineered human fetal hepatocyte organoids ● Timing 3 d**

▲ CRITICAL If a knockout line has been generated, this procedure is recommended to reveal potential (expected) phenotypes. If knockin lines have been generated and antibodies exist for the endogenous protein, we recommend co-staining for the endogenous protein to confirm localization of the tagged protein. Analysis of the localization of the endogenously tagged protein by microscopy often has higher resolution than antibody staining owing to antibodies having limited penetration into organoids.

▲ CRITICAL Organoid loss of ~30% of the starting material can be expected when staining organoids using this procedure, as a consequence of the multiple incubations and washes that require removal and replacement of the liquid surrounding the organoids. Therefore, it is critical to start with sufficient material to be able to obtain reliable and fully representative results. We recommend starting with two wells of a 12-well plate at least 3 d after organoid splitting so that organoids have formed of sufficient size (100–200 μm). It is also important to allow sufficient time for organoids to sink to the bottom of the tube by gravity or undertake appropriate centrifugation each time liquids need to be replaced (see Step 19A(iii) for specific guidance).

(i) Harvest two wells of a 12-well plate in 2 ml of ice-cold wash buffer in a 15-ml conical tube and top up the volume with ice-cold wash buffer to a total volume of 15 ml.
▲ CRITICAL Be very gentle when pipetting the organoids to avoid disruption of the structure.

(ii) Incubate the tube on ice for 30 min with occasional inverting of the tube to ensure complete dissolution of the BME.

(iii) Allow the organoids to sink to the bottom of the tube by gravity (this usually takes 10 min) or, alternatively, by low-speed centrifugation (100*g*).

(iv) Carefully aspirate the wash buffer, leaving the organoids in as little volume of the wash buffer as possible.

(v) Fix the organoids by adding 1 ml of 4% (vol/vol) formaldehyde and transfer the solution with the organoids to a 1.5-ml Safe-Lock tube. For gene knockin, depending on the tag used, avoid extensive fixation, as this might result in quenching of the endogenous fluorescence signal. We recommend 30 min–1 h fixation at room temperature on a rolling

wheel. For gene knockout, fixation can be performed for at least 1 h or, alternatively, overnight at 4°C.

! CAUTION When handling formaldehyde, be sure to execute all steps in a ventilated fume hood.

(vi) After fixation, ensure that the organoids are settled at the bottom of the tube and remove the fixation solution carefully with a P1000 pipette. Add 1 ml of PBS to the tube to wash the organoids. Upon settling of the organoids, carefully remove the PBS with a P1000 pipette and add 1 ml of PBS.

■ PAUSE POINT At this point, organoids can be stored in PBS for a few weeks at 4 °C. However, we recommend performing whole-mount staining as soon as possible.

(vii) To initiate whole-mount staining, carefully remove the PBS and permeabilize the organoids with 1 ml of permeabilization buffer. Incubate for 1 h at room temperature on a rolling wheel.

(viii) Allow the organoids to settle to the bottom of the tube (see Step 19A(iii) for details) and remove the permeabilization buffer. Block the organoids by adding 1 ml of blocking buffer. Incubate for 1 h at room temperature on a rolling wheel. In the meantime, prepare the primary antibody solution by diluting the specific desired primary antibodies in blocking buffer.

(ix) Allow the organoids to settle to the bottom of the tube, carefully remove the blocking buffer and add 200–500 µl of the primary antibody solution. Incubate overnight at 4°C on a rolling wheel.

(x) Allow the organoids to settle to the bottom of the tube and carefully remove the primary antibody solution. Wash the organoids with 1 ml of staining wash buffer. Incubate for 5 min at room temperature on a rolling wheel.

(xi) Repeat the washing steps as described in Step 19A(x) two more times. Be careful to allow the organoids to settle to the bottom each time to avoid loss of the organoids.

(xii) After the last washing step, carefully remove the staining wash buffer and add 200–500 µl of secondary antibody solution. Incubate for 2 h at room temperature on a rolling wheel.

(xiii) Allow the organoids to settle to the bottom of the tube and carefully remove the secondary antibody solution. Wash the organoids with 1 ml of staining wash buffer. Incubate for 5 min at room temperature on a rolling wheel.

(xiv) Repeat the washing steps as described in Step 19A(xiii) one more time.

(xv) Allow the organoids to settle to the bottom of the tube and then wash the organoids using DAPI-containing staining wash buffer. Incubate for 15 min at room temperature on a rolling wheel.

(xvi) Allow the organoids to settle to the bottom of the tube and then carefully remove the DAPI-containing staining wash buffer and add 1 ml of staining wash buffer. Incubate for 5 min at room temperature on a rolling wheel.

(xvii) Allow the organoids to settle to the bottom of the tube and then carefully remove the staining wash buffer and add 100 µl of preservation staining wash buffer to the organoids.

(xviii) Transfer the organoids to one well of a 96-well Sensoplate in the preservation staining wash buffer.

■ PAUSE POINT Organoids can be stored in the preservation staining wash buffer for 1–2 weeks.

(xix) Proceed to image, preferably using a confocal microscope. Post-imaging analysis can be performed using software such as ImageJ and Imaris.

(B) **Whole-mount staining of genome-engineered human liver ductal organoids**

(i) Because the human liver ductal organoids are cystic structures, they are very prone to break and collapse during harvesting. For this reason, whole-mount staining should be performed on a BME droplet containing the organoids in a plate.

(ii) Three to four days before staining, split the organoids as described in Step 1 of Procedure 2 and plate them in a 50-µl droplet in one well of a pre-heated 24-well Sensoplate.

▲ CRITICAL Because the Sensoplate is not a suspension culture plate, BME is more likely to spread and not form firm circular droplets. Optionally, a more concentrated BME resuspension solution can be used (BME:wash buffer 4:1 instead of 3:1).

(iii) On the day of staining, remove the medium and fix the organoids by adding 1 ml of 4% (vol/vol) formaldehyde to the well for 1 h.

! CAUTION When handling formaldehyde, be sure to execute all steps in a ventilated fume hood.

▲ CRITICAL During this step, the BME droplet tends to partly disintegrate, which will result in loss of ~20% of the organoids. Handle all subsequent steps with great care to avoid significant loss of the organoids.

(iv) Remove the formaldehyde and proceed with the washing and staining procedure as described for the human hepatocyte organoids (Steps 19A(vi–xvii), except that the incubation steps should be performed on a shaker, not a rolling wheel), using working volumes of ~500 µl for all the solutions.

(v) Before imaging, add 500 µl of preservation buffer to the well containing the stained organoids.

(vi) Proceed to imaging the organoids, preferably using a confocal microscope.

■ PAUSE POINT Organoids can be stored in the preservation staining wash buffer for 2–3 days.

(vii) Post-imaging analysis can be performed using software such as ImageJ and Imaris.

(C) **Live imaging and analysis of organoid knockin lines** ● Timing **3–5 d**

(i) Split the organoids as described in Steps 30–33 of Procedure 1 for human fetal hepatocyte organoids and Step 1 of Procedure 2 for human liver ductal organoids but placing the organoids into a single 50-µl BME droplet per well of a pre-heated 12-well glass-bottom plate or a pre-heated 24-well Sensoplate.

▲ CRITICAL Because these imaging plates are not suspension culture plates, BME is more likely to spread and not form firm circular droplets. Optionally, a more concentrated BME resuspension solution can be used (BME:wash buffer 4:1 instead of 3:1).

(ii) Incubate organoids in normal culture conditions until they reach the correct size and density for imaging (usually 2–4 d).

(iii) Image the organoids using a confocal microscope equipped with a temperature-controllable system or using a spinning disk. Optimize acquisition settings to avoid phototoxicity and bleaching of the endogenous fluorescence signal. The endogenous fluorescence signal driven by abundantly expressed genes usually allows imaging for at least 96 h without causing noticeable cytotoxicity and decrease in fluorescence signal. Labeling of cellular structures, such as the cell membrane and mitotic spindle, allows quantitative analysis and tracing of cell movements and cellular division in 3D over time. For these types of analyses, live-imaging files can subsequently be processed in ImageJ. This enables cell movement and cellular division of single cells to be traced manually and analyzed using custom-written Python scripts (e.g., as described in ref. [23]).

Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3 | Troubleshooting table

Step	Problem	Possible reason	Solution
Procedure 1, 27	Low viability of isolated hepatocytes	Incubation time with HEP digestion solution was too long and the tissue was overdigested	Digest the liver tissue for a shorter time
		Enzymatic activity of the HEP digestion solution was too high and the tissue was overdigested	Check carefully that the correct concentration of collagenase type IV was used to prepare the HEP digestion solution
		Mincing and pipetting was too vigourous	Mince the tissue more gently and avoid too much pipetting repetitions
Procedure 1, 36	Hepatocyte organoid line is contaminated with outgrowing cystic ductal-like organoids	Inclusion of white tree-like structures during the digestion steps	Manually remove the white tree-like structures present in the liver tissue during tissue dissection
		Inevitable outgrowth of cystic ductal-like organoids	Remove the cystic ductal-like organoids as soon as they appear in the cultures by manual picking
		Centrifugation speed was too high and so did not allow hepatocyte enrichment	Use the correct centrifugation at low speed

Table continued

Table 3 (continued)

Step	Problem	Possible reason	Solution
	Low yield of organoid formation after seeding	Poor quality or too little primary material	Process another sample
		Mistakes in the preparation of HEP isolation medium	Carefully check all medium component preparations and their correct storage and that all the components have been added to the HEP isolation medium
		Cells are too sparsely seeded	Recollect the seeded organoids, and re-plate them in a lower volume of BME resuspension solution
Procedure 1, 44	Hepatocyte organoid line stops growing or growth slows	Inherent capacity of the line owing to variability between donor material	Establish another hepatocyte organoid line
		Suboptimal seeding upon passaging at the wrong density	Recollect the seeded organoids and re-plate them in a lower volume of BME resuspension solution and allow the organoids to grow to a proper density before splitting
		Suboptimal passaging (shearing) of the organoids	Adjust the passaging procedure by less vigorous shearing
Box 1, 11	Poor recovery of the thawed frozen hepatocyte organoid line	Suboptimal freezing of hepatocyte organoids	Use the recommended commercial freezing medium
		Suboptimal thawing of hepatocyte organoids	Freeze down sufficient organoids in an appropriate volume to allow proper recovery
		Suboptimal outgrowth of hepatocyte organoids	Plate thawed organoids at the correct density (rather too dense than too sparse) and perform regular medium changes to remove the remaining DMSO
Procedure 1, 66; Procedure 2, 14	Poor transfection of hepatocytes and liver ductal cells	Inherent capacity of the line owing to variability between donor material	Pre-check the capacity of the lines to be transfected
		Plasmid size is too big	Whenever possible, use plasmids smaller than 10 kb
		Too low or too diluted concentration of DNA	Minimize the DNA volume while maximizing the DNA concentration
		Too much DNA is transfected	Adjust the amount of DNA to be transfected
Procedure 1, 67	Poor outgrowth of the hepatocytes into organoids after electroporation	Inherent capacity of the line owing to variability between donor material	Pre-check the capacity of the line to grow from single cells
		The single-cell dissociation step was performed too harshly and/or for too long	During the process of cell dissociation, check the cells at regular intervals and stop the dissociation as soon as most of the cells are single cells; do not prolong the digestion
		An insufficient number of cells were used for electroporation	Start with a higher number of cells
		Wrong electroporation settings used	Check that the correct electroporation settings have been used
		Wrong medium used for outgrowth	Check that HEP isolation medium has been used for cell recovery after isolation
Procedure 1, 67,77; Procedure 2, 19	No successful gene editing or lack of visibility of fluorescent tag expression in case of gene knockin	Inactive, poorly working sgRNAs	Design and test multiple sgRNAs
		Poorly accessible locus	Design and test novel sgRNAs targeting a different region of the gene (for instance, the second last exon for gene knockin or another early exon for gene knockout)
		For knockin, the target gene is not constitutively or sufficiently expressed	Check RNA and protein expression of the gene of interest to be tagged

Table continued

Table 3 (continued)

Step	Problem	Possible reason	Solution
Procedure 1, 89; Procedure 2, 37	Poor efficiency of clonal line establishment of genome-engineered cells into organoids	Organoids established when the wild-type line had been passaged a large number of times	Start as early as possible with genome engineering on the wild-type line to be used (especially relevant for human liver ductal organoid lines)
		Lethality of the introduced genome edit	Check whether heterozygously edited clones are instead growing out and viable, and use specific inhibitors, if possible, to screen for gene lethality
		The cell dissociation step to obtain clonal lines was performed too harshly and for too long	During the process of cell dissociation, check the cells at regular intervals and stop the dissociation as soon as most of the cells are single cells; do not prolong the digestion

Timing

All Procedures

Preparation for CRISPR–Cas9 genome engineering of organoid cultures from human liver

Component preparation, designing of sgRNAs takes ~1–2 h. Cloning of the sgRNAs, including screening and preparation of DNA, takes 4–5 d. Hands-on timing for this procedure is ~12–14 h

Procedure 1

Establishment of human fetal hepatocyte organoid cultures

Steps 1–27, collection and isolation of human hepatocytes from human fetal liver. Hands-on timing is usually 3–4 h for collection of the liver and dissociation into single hepatocytes. Hepatocyte isolation should be performed within 24 h from collection of the liver

Steps 28–33, hands-on timing for seeding of human fetal hepatocytes for organoid formation is ~1–2 h. This procedure should be immediately performed after Steps 1–27

Steps 34–44, establishment and culture of human fetal hepatocyte organoid lines. This phase can take ~6–8 weeks. A hepatocyte organoid line can be considered established from five passages onwards. Hands-on timing is ~3-4 h per week for medium change and splitting

Box 1 Freezing and thawing of human fetal hepatocyte organoids. Freezing of the organoids is recommended as soon as the organoid line has been establishe (i.e., at Step 44). Hands-on timing for freezing is ~1 h and for thawing is ~2 h

Genome engineering of human fetal hepatocyte organoids

Step 45, hands-on timing for preparation of the DNA mixture for transfection takes ~30 min–1 h

Steps 46–57, hands-on timing for preparation of the human fetal hepatocyte organoids for electroporation takes ~1–2 h

Steps 58–65, hands-on timing for electroporation of the human fetal hepatocyte organoids with the DNA mixture and subsequent organoid seeding takes ~1–2 h

Steps 66–89, genome-engineered human hepatocyte organoid line establishment. This phase can take ~6–8 weeks

Steps 68–77, (optional) FACS of human fetal hepatocyte organoids. Hands-on timing for preparing cells, FACS and subsequent plating of single cells is ~4–5 h

Steps 78–79, (optional) drug selection. This step can take ~2 weeks depending on the selection method. Hands-on timing is ~6–8 h

Steps 80–89, hands-on timing for picking of organoids is ~4 h

Procedure 2

Establishment of human liver ductal organoid lines

Component preparation, establishment of human liver ductal organoid lines from adult liver biopsies takes 3–4 weeks to scale up to sufficient material to be used for genome engineering of the organoids. Refer to Broutier et al.[31] for detailed timing

Genome engineering of human liver ductal organoids

Step 1, preparation of the human liver organoids should be performed 3–4 d before electroporation. Hands-on timing is ~1–2 h

Steps 2 and 3, hands-on timing for preparation of the DNA mixture for transfection takes ~30 min–1 h

Steps 4–7, hands-on timing for preparation of the capillaries and their mounting takes ~30 min–1 h
Steps 8–13, hands-on timing for electroporation of the human liver ductal organoids with the DNA mixture takes ~2–4 h
Steps 14–37, establishment of genome-engineered human liver ductal organoid lines. This phase can take~6–8 weeks
Steps 32 and 33, (optional) drug selection. This step can take ~2 weeks depending on the selection method. Hands-on timing is ~6–8 h
Steps 34–37, hands-on timing for picking of organoids and subsequent seeding is ~4 h
Procedure 3
Genotyping of genome-engineered organoids from human liver
Steps 1–16, the entire procedure to genotype takes ~3–5 d. Hands-on timing for DNA isolation, PCR purification, subcloning (optional) and sequencing is ~6–24 h
Multiplexing gene knockin with gene knockout or generation of multi/sequential gene knockin/knockout organoid lines
Steps 17–18, this phase can take ~6–8 weeks. Applying drug selection (optional), picking, organoid seeding and medium changes is ~48–96 h distributed over the whole phase
Downstream applications of genome-engineered organoids from human liver:
Whole-mount staining of genome-engineered organoid knockin and knockout lines.
Step 19, options A and B, the entire procedure for both human fetal hepatocyte organoids and human liver ductal organoids takes 3 d. Hands-on timing, including preparation of the sample, imaging and post-imaging analysis, takes ~12–48 h
Live imaging and analysis of organoid knockin lines.
Step 19, option C, live imaging takes ~3–5 d. Organoids should be plated 2–4 d before imaging. Hands-on timing for organoid plating, imaging and post-imaging analysis takes ~3–12 h

Anticipated results

This protocol, if executed correctly and using the appropriate reagents, should lead to establishment of 3D hepatocyte organoid lines from human fetal liver. Moreover, this protocol describes the procedures to efficiently generate gene knockin and gene knockout for both organoid cultures from human liver (i.e., adult human liver ductal organoids and human fetal hepatocyte organoids). Availability of fresh material is considered a prerequisite for successful line generation. High-quality growth factors and quality-checked RSPO1-CM and BME batches are important assets in the establishment of the lines. Starting from fresh liver material to successful and stable hepatocyte organoid line establishment should take ~2 months and might occasionally require picking out outgrowing cystic ductal-like organoids from the culture (Fig. 2c,d). We have so far tested organoid line formation from 20 donors with a success rate of 70% (i.e., 14 lines established, defined as a line capable of being passaged for more than five passages). Human fetal hepatocyte organoid cultures should present as dense organoids, either spherical or in variable, more cauliflower-like, structures, sometimes with small internal lumina (Figs. 2c and 5a). Hepatocytes within the organoid should display a typical cuboidal shape, with a small percentage being binucleated cells, as, for example, revealed by B-CAT staining marking the cell membrane (Fig. 5a). The hepatocyte organoid morphologies can be easily distinguished from the cystic appearance of the human liver ductal organoids (Fig. 5b). Immunofluorescent stainings of the hepatocyte organoid cultures should reveal abundant, uniform expression of typical hepatocyte markers, including ALB and A1AT (*SERPINA1*). Staining for a bile canalicular marker (e.g., MRP2) should highlight the presence of an intact, structured bile canalicular network (Fig. 5a). In our experience, established human fetal hepatocyte organoid lines can be stably cultured for over 40 passages with a 7–12-d split ratio of 1:3-1:5; this has meant that some have grown for over 1 year in culture. Transcriptomic profiling of the organoid cultures over multiple passages has demonstrated stability of gene expression, and there are some similarities in gene expression profile when compared to PHH[20] (Supplementary Fig. 1). Additionally, lines established from donors of different gestational age demonstrate some similarities in mRNA expression for some hepatic markers (Supplementary Fig. 2).

Adult human liver ductal organoids can be stably cultured for ~15–20 passages with a 5- to 10-d split ratio of 1:4–1:6, lasting for ~6 months[14]. Immunofluorescent stainings of human liver ductal organoids should detect typical biliary markers, such as KRT7 or KRT19, and there should not be expression of hepatocyte markers, such as ALB (Fig. 5b). Staining for polarity markers,

such as ZO-1, reveals the overall proper organization and polarity of the ductal cells within the organoid (Fig. 5b).

We have achieved robust genome engineering of both the human fetal hepatocyte and the adult liver ductal organoids (Figs. 3,4 and 5c,d and Table 2). The transfection efficiency and the tolerance of the lines for electroporation, and, therefore, gene editing, is, however, donor dependent for both organoid culture systems. We have not determined, as yet, why and have not observed any correlation with the age and sex of the donor. The rate of establishment of clonal genome-engineered organoid lines does depend on the outgrowth from single cells. Proper and delicate handling of the organoids is critical for robust genome engineering. In particular, the optimal duration of dissociation and appropriate washing of the cells to remove any residue of dissociation enzymes are critical when dissociating organoids into single cells or small clumps of cells. We have generated genome-engineered organoids from the vast majority of the lines we have tested. We have generated genome-engineered lines from a total of five hepatocyte lines and 20 liver ductal lines but found clonal outgrowth, and, thus, the amount of desired genome-engineered lines obtained varied between 5 and 100 clones within different donor lines and experiments (Table 2). We, therefore, recommend testing the capacity of each organoid line to regrow from single cells before using a particular line for genome engineering. A suitable line should be able to regrow ~60% of the single cells seeded. This process takes time; obtaining clonal genome-engineered organoid lines typically takes 2–3 months from initial transfection.

Genotyping is a must to confirm proper genome engineering. When we have used gene knockin using CRISPR-HOT, all fluorescence-positive lines had the fluorescent tag precisely integrated in the desired location[23]. Antibody staining should be used to confirm that gene tagging does not interfere with localization of the native protein. When we have compared antibody staining and endogenous tagging, we have found that tagging of the endogenous protein reveals better resolution of fine details. As a consequence, there is not always an exact overlap between the two signals. We hypothesize that this could partially be due to limited antibody penetration or antibody specificity. To date, we have not observed protein mislocalization (including for the structural proteins encoded by the *TUBB* and *CDH1* genes); however, such effects could be gene dependent.

For any genome-engineered organoid line, it is important to critically observe the behavior of the line and to perform basic organoid characterization experiments (e.g., evaluation of typical cell marker expression as shown in Fig. 5). When undertaking gene knockin using CRISPR-HOT, we have not observed changes in behavior of these lines when compared to the wild-type parental line in terms of growth speed, morphology and passaging number. For gene knockout, there might be changes in these parameters owing to the specific gene targeted. In such cases, it is important to assess the robustness of the phenotype over multiple clones and in organoid cultures derived from different donors.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

References

1. Khetani, S. R. & Bhatia, S. N. Microscale culture of human liver cells for drug development. *Nat. Biotechnol.* **26**, 120–126 (2008).
2. Bell, C. C. et al. Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. *Sci. Rep.* **6**, 25187 (2016).
3. Xiang, C. et al. Long-term functional maintenance of primary human hepatocytes in vitro. *Science* **364**, 399–402 (2019).
4. Jang, K. J. et al. Reproducing human and cross-species drug toxicities using a Liver-Chip. *Sci. Transl. Med.* **11**, eaax5516 (2019).
5. Takebe, T. et al. Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* **499**, 481–484 (2013).
6. Wu, F. et al. Generation of hepatobiliary organoids from human induced pluripotent stem cells. *J. Hepatol.* **70**, 1145–1158 (2019).
7. Mun, S. J. et al. Generation of expandable human pluripotent stem cell-derived hepatocyte-like liver organoids. *J. Hepatol.* **71**, 970–985 (2019).
8. Wang, Q. et al. Generation of human hepatocytes from extended pluripotent stem cells. *Cell Res.* **30**, 810–813 (2020).

9. Takebe, T. et al. Massive and reproducible production of liver buds entirely from human pluripotent stem cells. *Cell Rep.* **21**, 2661–2670 (2017).
10. Huang, P. et al. Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. *Cell Stem Cell* **14**, 370–384 (2014).
11. Nakamori, D., Akamine, H., Takayama, K., Sakurai, F. & Mizuguchi, H. Direct conversion of human fibroblasts into hepatocyte-like cells by ATF5, PROX1, FOXA2, FOXA3, and HNF4A transduction. *Sci. Rep.* **7**, 16675 (2017).
12. Xie, B. et al. A two-step lineage reprogramming strategy to generate functionally competent human hepatocytes from fibroblasts. *Cell Res.* **29**, 696–710 (2019).
13. Sun, L. et al. Modelling liver cancer initiation with organoids derived from directly reprogrammed human hepatocytes. *Nat. Cell Biol.* **21**, 1015–1026 (2019).
14. Huch, M. et al. Long-term culture of genome-stable bipotent stem cells from adult human liver. *Cell* **160**, 299–312 (2015).
15. Zhang, K. et al. In vitro expansion of primary human hepatocytes with efficient liver repopulation capacity. *Cell Stem Cell* **23**, 806–819 (2018).
16. Kim, Y. et al. Small molecule-mediated reprogramming of human hepatocytes into bipotent progenitor cells. *J. Hepatol.* **70**, 97–107 (2019).
17. Katsuda, T. et al. Generation of human hepatic progenitor cells with regenerative and metabolic capacities from primary hepatocytes. *eLife* **8**, e47313 (2019).
18. Fu, G. B. et al. Expansion and differentiation of human hepatocyte-derived liver progenitor-like cells and their use for the study of hepatotropic pathogens. *Cell Res.* **29**, 8–22 (2019).
19. Peng, W. C. et al. Inflammatory cytokine TNFα promotes the long-term expansion of primary hepatocytes in 3D culture. *Cell* **175**, 1607–1619 (2018).
20. Hu, H. et al. Long-term expansion of functional mouse and human hepatocytes as 3D organoids. *Cell* **175**, 1591–1606 (2018).
21. Hendriks, D., Clevers, H. & Artegiani, B. CRISPR-Cas tools and their application in genetic engineering of human stem cells and organoids. *Cell Stem Cell* **27**, 705–731 (2020).
22. Artegiani, B. et al. Probing the tumor suppressor function of BAP1 in CRISPR-engineered human liver organoids. *Cell Stem Cell* **24**, 927–943 (2019).
23. Artegiani, B. et al. Fast and efficient generation of knock-in human organoids using homology-independent CRISPR–Cas9 precision genome editing. *Nat. Cell Biol.* **22**, 321–331 (2020).
24. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262–265 (2009).
25. Sato, T. et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762–1772 (2011).
26. Bartfeld, S. et al. In vitro expansion of human gastric epithelial stem cells and their responses to bacterial infection. *Gastroenterology* **148**, 126–136 (2015).
27. Schutgens, F. et al. Tubuloids derived from human adult kidney and urine for personalized disease modeling. *Nat. Biotechnol.* **37**, 303–313 (2019).
28. Sachs, N. et al. Long-term expanding human airway organoids for disease modeling. *EMBO J.* **38**, e100300 (2019).
29. Sekine, S., Gutierrez, P. J., Lan, B. Y., Feng, S. & Hebrok, M. Liver-specific loss of beta-catenin results in delayed hepatocyte proliferation after partial hepatectomy. *Hepatology* **45**, 361–368 (2007).
30. Gao, S. et al. Ex vivo gene delivery to hepatocytes: techniques, challenges, and underlying mechanisms. *Ann. Biomed. Eng.* **40**, 1851–1861 (2012).
31. Broutier, L. et al. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. *Nat. Protoc.* **11**, 1724–1743 (2016).
32. Artegiani, B., Lange, C. & Calegari, F. Expansion of embryonic and adult neural stem cells by in utero electroporation or viral stereotaxic injection. *J. Vis. Exp.* **68**, 4093 (2012).
33. Fujii, M., Matano, M., Nanki, K. & Sato, T. Efficient genetic engineering of human intestinal organoids using electroporation. *Nat. Protoc.* **10**, 1474–1485 (2015).
34. Drost, J. et al. Sequential cancer mutations in cultured human intestinal stem cells. *Nature* **521**, 43–47 (2015).
35. Drost, J. et al. Use of CRISPR-modified human stem cell organoids to study the origin of mutational signatures in cancer. *Science* **358**, 234–238 (2017).
36. Matano, M. et al. Modeling colorectal cancer using CRISPR–Cas9-mediated engineering of human intestinal organoids. *Nat. Med.* **21**, 256–262 (2015).
37. Fumagalli, A. et al. Genetic dissection of colorectal cancer progression by orthotopic transplantation of engineered cancer organoids. *Proc. Natl Acad. Sci. USA* **114**, E2357–E2364 (2017).
38. Schwank, G. et al. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* **13**, 653–658 (2013).
39. Cortina, C. et al. A genome editing approach to study cancer stem cells in human tumors. *EMBO Mol. Med.* **9**, 869–879 (2017).
40. Sugimoto, S. et al. Reconstruction of the human colon epithelium in vivo. *Cell Stem Cell* **22**, 171–176 (2018).
41. Shimokawa, M. et al. Visualization and targeting of $LGR5^{+}$ human colon cancer stem cells. *Nature* **545**, 187–192 (2017).

42. Haapaniemi, E., Botla, S., Persson, J., Schmierer, B. & Taipale, J. CRISPR–Cas9 genome editing induces a p53-mediated DNA damage response. *Nat. Med.* **24**, 927–930 (2018).
43. Ihry, R. J. et al. p53 inhibits CRISPR–Cas9 engineering in human pluripotent stem cells. *Nat. Med.* **24**, 939–946 (2018).
44. Schiroli, G. et al. Precise gene editing preserves hematopoietic stem cell function following transient p53-mediated DNA damage response. *Cell Stem Cell* **24**, 551–565 (2019).
45. Yeh, C. D., Richardson, C. D. & Corn, J. E. Advances in genome editing through control of DNA repair pathways. *Nat. Cell Biol.* **21**, 1468–1478 (2019).
46. Betermier, M., Bertrand, P. & Lopez, B. S. Is non-homologous end-joining really an inherently error-prone process? *PLoS Genet.* **10**, e1004086 (2014).
47. Lackner, D. H. et al. A generic strategy for CRISPR–Cas9-mediated gene tagging. *Nat. Commun.* **6**, 10237 (2015).
48. Suzuki, K. et al. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature* **540**, 144–149 (2016).
49. Schmid-Burgk, J. L., Honing, K., Ebert, T. S. & Hornung, V. CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism. *Nat. Commun.* **7**, 12338 (2016).
50. Guo, T. et al. Harnessing accurate non-homologous end joining for efficient precise deletion in CRISPR/Cas9-mediated genome editing. *Genome Biol.* **19**, 170 (2018).
51. Kleinstiver, B. P. et al. Engineered CRISPR–Cas9 nucleases with altered PAM specificities. *Nature* **523**, 481–485 (2015).
52. Hu, J. H. et al. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* **556**, 57–63 (2018).
53. Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A. & Liu, D. R. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **533**, 420–424 (2016).
54. Gaudelli, N. M. et al. Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. *Nature* **551**, 464–471 (2017).
55. Kuscu, C. et al. CRISPR-STOP: gene silencing through base-editing-induced nonsense mutations. *Nat. Methods* **14**, 710–712 (2017).
56. Anzalone, A. V. et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* **576**, 149–157 (2019).
57. de Lau, W. et al. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature* **476**, 293–297 (2011).
58. Korinek, V. et al. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC−/− colon carcinoma. *Science* **275**, 1784–1787 (1997).
59. Ran, F. A. et al. Genome engineering using the CRISPR–Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013).
60. Labun, K. et al. CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Res.* **47**, W171–W174 (2019).

Acknowledgements

We thank H. Gehart for providing fluorescent images of human liver ductal organoids; S. van der Brink for RSPO1-conditioned medium production; and L. Huang and L. Luciana for critically reading the manuscript. We acknowledge all of the anonymous tissue donors. This work is part of the Oncode Institute, which is partly financed by the Dutch Cancer Society. D.H. is supported by an SSMF postdoctoral fellowship (P19-0074).

Author contributions

Conceptualization: D.H. and B.A.; methodology: D.H., B.A. and H.H; formal analysis: D.H. and B.A.; investigation: D.H. and B.A.; resources: S.C.v.S.L. and H.C.; data curation: D.H. and B.A.; writing—original draft: D.H., B.A. and S.C.v.S.L.; writing—review and editing: D.H., B.A. and H.C.; visualization: D.H. and B.A.; project administration: D.H. and B.A.; and funding acquisition: H.C.

Competing interests

H.C. holds several patents on organoid technology.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41596-020-00411-2.

Correspondence and requests for materials should be addressed to D.H., B.A. or H.C.

Peer review information *Nature Protocols* thanks Ömer Yilmaz and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 18 February 2020; Accepted: 17 September 2020;
Published online: 27 November 2020

Related links

Key references using this protocol

Hu, H. et al. *Cell* 175, 1591–1606 (2019): https://doi.org/10.1016/j.cell.2018.11.013
Artegiani., B. et al. *Cell Stem Cell* 24, 927–943 (2019): https://doi.org/10.1016/j.stem.2019.04.017
Artegiani, B. et al. *Nat Cell Biol.* 22, 321–331 (2020): https://doi.org/10.1038/s41556-020-0472-5