CHAPTER

Pluripotent stem cell-derived cholangiocytes and cholangiocyte organoids

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Chapter outline

1 Introduction....70
2 Physiology of cholangiocytes....71
2.1 Small and large cholangiocytes....72
2.2 Membrane transporters....72
3 Biliary differentiation during development....74
3.1 Intrahepatic cholangiocyte progenitor differentiation during development....74
3.2 Hepatoblast differentiation into cholangiocytes during development....74
4 Human cholangiocyte sources for organoids....75
5 Applications of pluripotent stem cell-derived cholangiocytes and cholangiocyte organoids....76
5.1 Disease modeling and drug screening....76
5.2 Tissue engineering....77
5.3 Regenerative medicine....78
6 Overview of the protocol....78
7 Step-by-step methods....80
7.1 hiPSC culture....80
7.1.1 Materials and reagents....80
7.1.2 Protocol....80
7.2 Coating gelatin plates for hiPSC differentiation....81
7.2.1 Materials and reagents....81
7.2.2 Protocol....81
7.3 Differentiation of hiPSCs into hepatoblasts....81
7.3.1 Material and reagents....81
7.3.2 Protocol....82

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7.4 Coating collagen plates for hepatoblast differentiation into cholangiocytes......83
7.4.1 Material and reagents......83
7.4.2 Protocol......83
7.5 Differentiation of hepatoblasts into cholangiocytes......84
7.5.1 Material and reagents......84
7.5.2 Protocol......84
7.6 Characterization of cholangiocyte organoids......86
7.6.1 Immunofluorescence......86
7.6.2 Functional test of bile acid transport by hiPSC-cholangiocyte organoids using cholyl-lysyl-fluorescein (CLF)......89
8 Conclusion......**89**
Acknowledgments......**89**
References......**90**

Abstract

The development of protocols for pluripotent stem cell (PSC) differentiation into cholangiocytes and cholangiocyte organoids in three-dimensional structures represent a huge advance in both research and medical fields because of the limited access to primary human cholangiocytes and the potential bias induced by animal models used to study cholangiopathies *in vivo*. PSC-derived cholangiocyte organoids consisting of either cysts with luminal space or branching tubular structures are composed of cells with apico-basal polarity that can fulfill cholangiocyte functions like the transport of bile salts. Several protocols of PSC differentiation have already been published but we added to the detailed protocol we describe here some notes or advice to facilitate its handling by new users. We also propose detailed protocols to carry out some of the characterization analyses using immunofluorescence to study the expression of specific markers and a functionality test to visualize bile acid transport using cholyl-lysyl-fluorescein (CLF).

1 Introduction

The human liver is responsible for numerous functions like the serum detoxification, synthesis of serum proteins, various metabolic activities, bile acid production and even participates in immune regulation. These functions are mainly fulfilled by two types of epithelial cells: the hepatocytes and the cholangiocytes. The hepatocytes represent about 95% of parenchymal cells and perform the majority of essential liver functions. Here we will focus on cholangiocytes, which regulate bile secretion, modify its components and form a biliary tree to collect and transport the bile from the hepatocytes to the intestine.

The bile is a complex fluid with basic pH, composed of water, electrolytes and organic molecules like biliary acids, cholesterol, phospholipids, bilirubin and proteins. Biliary acids are secreted by hepatocytes into a biliary canaliculi network that come out in the canals of Hering, where the cholangiocytes form the peripheral ductules and bile ducts of the biliary tree. Human bile duct organogenesis and biliary disorders are challenging to study because of the difficulty isolating and amplifying functional human primary cholangiocytes. To overcome this problem, new cell sources have been developed, such as pluripotent stem cell-derived cholangiocytes, which open the door to numerous exciting applications like disease modeling and tissue regeneration (Zhao et al., 2009). To better mimic *in vitro* the *in vivo* cell environment, 3D-culture techniques have been developed to obtain cholangiocytes organoids. By definition, an organoid is a self-organizing 3D structure that mimics some of the *in vivo* functions of an organ. For cholangiocytes, it consists in the formation of 3D cysts or branching tubular structures with luminal space as well as apico-basal polarity that can fulfill cholangiocyte functions.

The focus of this chapter is to describe in detail the protocol we have established for the generation of human cholangiocyte organoids derived from human induced pluripotent stem cells (hiPSCs). In addition, we will first discuss the physiology and function of these cells as well as their formation during human development in order to better understand the different steps of the differentiation protocol.

2 Physiology of cholangiocytes

The biliary tree can be divided into two parts: the extrahepatic and intrahepatic (Kanno, LeSage, Glaser, Alvaro, & Alpini, 2000). Here we will focus on intrahepatic biliary tree initially defined as “Progressively larger interconnecting sets of tubular structures lined by cholangiocytes” (Alpini et al., 1997).

Cholangiocytes are polarized biliary epithelial cells that represent approximately 3% of liver cells and line the bile ducts of the biliary tree. Even if they represent a low percentage of the total liver cells, cholangiocytes have a crucial role in liver homeostasis as they modify the composition of the bile produced by the hepatocytes by altering the water and solute content so that it does not injure the organ. They have a large cytoskeleton in the form of a network of Cytokeratins 7 and 19 (CK7 and CK19), a basal membrane and present tight junctions between cells and microvilli that project into the lumen of the bile duct (Glaser, 2006). They also possess a unique primary cilia, a sensory organelle on the cell apical surface, that plays an important role in modulating the secretory and proliferative functions of cholangiocytes (Huang et al., 2006). The cilia are ideally positioned to act as mechano- or chemosensors to detect changes in bile flow and bile composition respectively (LaRusso & Masyuk, 2011).

2.1 Small and large cholangiocytes

The intrahepatic biliary tree can be classified by size, from bile ductules (<15 μM) to hepatic ducts (>800 μm). The large ducts lined by 8–15 cells in rodents and up to 40 in humans and small ducts by 4–5 cells in both species (Alpini et al., 1997; Buisson, Jeong, Kim, & Choi, 2019). The morphological characteristics and functional properties of cholangiocytes previously mentioned are not identical throughout the biliary tree and vary according to the diameter of the channels.

Indeed, several studies have shown that cholangiocyte size, morphology, proliferation, activity and function differ depending on their position along the intrahepatic biliary tree. Small cholangiocytes are approximately 9 μm diameter in rat (<15 μm in human) and line the interlobular bile ducts, ductules and the canals of Hering. They express CK7, CK19, NCAM, and the aquaporin-1 (AQP1) but not the secretin receptor (SCTR), the somatostatin receptor 2 (SSTR2), the anion exchanger Cl-/HCO3-(AE2) and the cystic fibrosis receptor (CFTR) (Fig. 1). In terms of proliferation, cholangiocytes are normally quiescent but small ones can occasionally proliferate in response to different events such as injury caused by drug administration. Large cholangiocytes are approximately 15 μm diameter in rat (>15 μm in human) and line large ducts, including the extrahepatic bile duct. They have a columnar morphology (Cardinale et al., 2012) and are quiescent cells expressing SCTR, SSTR2, AE2 and CFTR (Alpini et al., 1997; Glaser, 2006; Maroni et al., 2015).

Functionally, large cholangiocytes are more susceptible to damage compared to small cholangiocytes (LeSage et al., 1999). The presence of the antiapoptotic protein Bcl-2 (Charlotte et al., 1994) in small cholangiocytes can explain this difference in terms of resistance to liver injury or toxins.

2.2 Membrane transporters

Cholangiocytes and hepatocytes line the Canal of Hering, which represents the physiological link between the canalicular system (hepatocytes) and the biliary system (cholangiocytes) (Fig. 1). The membrane transporters ensuring the formation of bile are now largely identified. The export of bile to the canalicular space is provided by several canalicular membrane transporters present on the apical membrane of hepatocytes that belonging to the superfamily ABC (ATP binding cassette) (Meier & Stieger, 2002). On cholangiocyte membranes, several receptors have been identified. On their apical membrane, where the cholangiocytes are exposed to high concentrations of bile acids, the selective transporter for bile acids, the apical sodium-dependent bile salt transporter (ASBT), is expressed. Because of the cytotoxic effect of intracellular accumulation of bile acids, the bile acid efflux is allowed by transport systems present on the cholangiocyte basolateral membrane that unidirectionally move bile acids from the bile duct to the bloodstream through the t-ASBT (truncated ASBT), multidrug resistance protein 3 (MRP3/ABCB4) and the organic solute transporter Ost alpha-Ost beta. Alternatively, modified bile acids are excreted in the bile flow through MRP2 export pump. The major membrane transporters of both hepatocytes and cholangiocytes have been represented in Fig. 1.

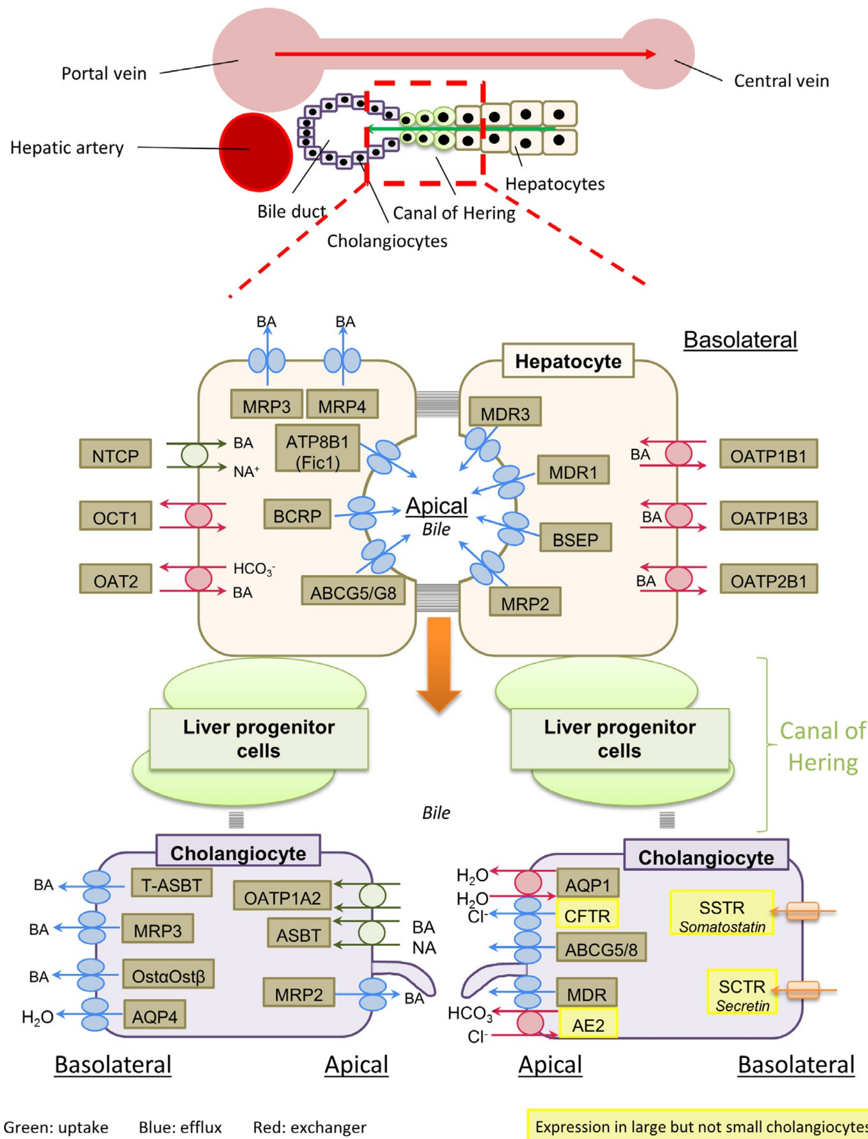

FIG. 1

Localization of uptake and efflux transport proteins in polarized hepatocytes and cholangiocytes. The schematic representation on the top of the figure allows to localize the transition zone between hepatocytes and cholangiocytes in the liver lobule (canal of Hering). The localization and orientation of uptake and efflux transporters are shown. Transporters expressed only in large but not small cholangiocytes are specified.

3 Biliary differentiation during development

The extrahepatic and intrahepatic bile ducts develop differently and at different times during embryogenesis. The epithelial cells lining the extrahepatic biliary tract have a common developmental origin with the pancreas while intrahepatic duct cholangiocytes, that develop later, derive from progenitor cells around the ductal plate in the periportal regions (Antoniou et al., 2009; Spence et al., 2009; Strazzabosco & Fabris, 2012). As previously mentioned, we will now focus on intrahepatic bile ducts.

3.1 Intrahepatic cholangiocyte progenitor differentiation during development

Liver derives from the endoderm, one of three primary germ layers that emerge during gastrulation. Hepatic specification of the endoderm results in the formation of a hepatic bud at embryonic day 9 in mice and 24 in human, and takes place in a region of the ventral foregut located near the cardiac mesoderm and a mesenchymal tissue called *septum transversum*. The cardiac mesoderm will secrete cues to induce liver from the Fgf family (Fibroblast Growth Factor 1 and 2) (Serls, Doherty, Parvatiyar, Wells, & Deutsch, 2005) and the *septum transversum* secretes ligands from the Bmp family (Bone Morphogenetic Protein 2 and 4), with Bmp being regulated by Gata4 transcription factor (Rossi, Dunn, Hogan, & Zaret, 2001). The role of the Wnt/β-catenin pathway has been also described at early stages of liver development, showing that the suppression of the Wnt pathway is necessary for the endoderm to become hepatic tissue (McLin, Rankin, & Zorn, 2007).

After liver specification, hepatoblasts continue proliferating to allow the growth of the liver bud. This proliferation is controlled and regulated by many factors, including Hgf (Hepatocyte Growth Factor) expressed by the mesenchymal cells of the *septum transversum*, endothelial cells and hepatoblasts themselves. Another factor involved in hepatoblast proliferation is the retinoic acid which acts on the Wt1 expression (Wilms' tumor suppressor gene) necessary for the liver development in mice (Ijpenberg et al., 2007).

Hepatoblasts, proliferating and migrating in the *septum transversum* mesenchyme to form the liver bud (Matsumoto, 2001), are bipotent, meaning that they can either differentiate into hepatocytes or cholangiocytes depending on their location. The specification process during cholangiocyte differentiation is thus a balance between the induction of cholangiocyte-specific genes and the repression of hepatocyte-specific genes.

3.2 Hepatoblast differentiation into cholangiocytes during development

The intrahepatic bile duct is formed after the hepatic mass has already begun to expand and the lobes of the liver have formed. After about 8 weeks of gestation in humans, the hepatoblasts form a temporary structure around the portal vein

consisting of a monolayer of cholangiocyte precursors, called the ductal plate (Antoniou et al., 2009). Lumens will then focally appear, lined on the portal side by biliary cells that express typical markers such as Sox9, Osteopontin (Opn) and Ck19 and by hepatoblasts on the parenchymal side. When growing along the portal vein toward the periphery of the liver, these structures will then mature, becoming entirely lined by biliary cells with apico-basal polarity (Lemaigre, 2020).

One of the first pathways that lead to the ductal plate formation is the Notch signaling pathway which induces Sox9 expression, the most specific and early marker of biliary cells. Notch signaling contributes not only to the initiation of the biliary development but also to the duct morphogenesis. Another important pathway involved in the biliary commitment of hepatoblasts is the Tgfβ signaling, that acts as a gradient with a high activity near the portal vein and a lower activity in the parenchyma to induce cholangiocyte differentiation and to inhibit hepatocyte differentiation (Clotman, 2005). Finally, in addition to Notch and Tgfβ, members of the Wnt family also regulate differentiation of hepatoblasts to biliary cells, like Wnt3A, and improve the proliferation and survival capacity of bile duct cells (Hussain et al., 2004).

The final intrahepatic bile duct is thus composed of large ducts running along the portal veins and small channels formed by cells that form connections to the canals of Hering to drain the bile out of the organ. The bile secretion starts around the 16th week of gestation in human, while the morphogenesis of the biliary system ends only at the 28th week. The final maturation of the biliary canals will continue few years after birth (Strazzabosco & Fabris, 2012).

4 Human cholangiocyte sources for organoids

As for all cell types of the body, the first source of cells that can be used in research are primary cells such as rat cholangiocytes, which have been used in several studies. However, there are two major limitations to the use of primary cells for human cholangiocyte studies. The first one is their very poor availability because of the limited access to liver and because there is a paucity of cholangiocytes harvested from donor livers. The second limitation is the difficulty, as for hepatocytes, to maintain the function of primary cholangiocytes *in vitro*.

Several alternatives have thus been evaluated like the use of the HepaRG cell line, an immortalized hepatic progenitor cell line isolated from a patient suffering from a cholangiocarcinome (Gripon et al., 2002). As hepatic progenitor cells, HepaRG can be differentiated into cholangiocytes (Dianat et al., 2014). However, because of the tumorigenic source of the cells, they cannot be used in therapeutic applications.

In 2014, our laboratory developed a protocol for the differentiation of pluripotent stem cells into functional cholangiocytes (Dianat et al., 2014). Since then, several publications reported the use of either embryonic stem cells or pluripotent stem cells to differentiate functional human cholangiocytes (De Assuncao et al., 2015; Ogawa et al., 2015; Sampaziotis et al., 2015, 2017).

Initially based on a first step of two-dimensional culture, these differentiation protocols further allow the formation in Matrigel of 3D-structures called "cholangiocyte organoids" as they fulfill some of the essential biliary functions. These 3D cultures lead to small cystic or tubular structures with a central lumen that resemble native bile ducts and are associated with improved cholangiocyte function and enhanced growth of the derived cholangiocytes.

5 Applications of pluripotent stem cell-derived cholangiocytes and cholangiocyte organoids

5.1 Disease modeling and drug screening

Cholangiocytes can be the target of different groups of biliary diseases called cholangiopathies including inherited disorders such as Alagille syndrome (AGS) and cystic fibrosis, autoimmune disorders such as primary sclerosing cholangitis (PSC), some ciliopathies such as Polycystic liver disease (PLD), infections such as cholangitis, drug-induced injury and finally ischemic injury.

Several mouse models have been developed to better investigate the mechanisms of these diseases but investigations on human cells are still required because of the potential interspecies differences (Mariotti et al., 2019). The use of cholangiocytes and cholangiocyte organoids differentiated from patient human induced pluripotent stem cells (hiPSCs) allow the *in vitro* modeling of these pathologies to better understand their mechanistic and development. Alagille syndrome (AGS) for example, is the most common duct malformation caused by a mutation in *JAG-1* or *NOTCH2* (Li et al., 1997; McDaniell et al., 2006). This disease not only affects the liver but can also affect the cardiac, renal, skeletal and ophthalmic systems (Kim, Yang, Paik, Choe, & Paik, 2017). The use of patient-specific hiPSCs allows the development of an *in vitro* model of the disease and to reproduce its phenotype in a dish highlighted by the lack of lumen in the differentiated cysts (Guan et al., 2017; Sampaziotis et al., 2015).

Another common cholangiopathy is the Cystic fibrosis (*CF*), an autosomal recessive disorder caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator gene (*CFTR*). This leads to a reduced intraluminal chloride secretion and an increased bile viscosity. The phenotype of this disease has been reproduced *in vitro* using hiPSCs derived from skin fibroblasts of patients, showing a defect in the chloride transfer in the organoid lumen (Ogawa et al., 2015; Sampaziotis et al., 2015). This model has also been used to test an experimental compound, the VX809, already used in phase 2 clinical trials for *CF* lung disease (Ogawa et al., 2015; Sampaziotis et al., 2015). Results showed that VX809 increases CFTR function and improves the intraluminal fluid secretion, suggesting an extended therapeutic effect of this compound on *CF* liver diseases.

Polycystic liver disease (PLD) is a rare inherited disorder estimated to affect around 1 in 100,000 people and characterized by the progressive growth of cysts of various sizes and multiple cystic lesions in the liver. People affected by this disease tend to develop numerous cysts. Surgical interventions are performed to

reduce the size of the larger cysts (>5 cm); however, liver transplantation represents the only definitive treatment, but is used in only the most severe cases. Polycystic diseases are multiorgan disorders and if numerous mouse models of polycystic kidneys with PLD have been developed (Wilson, 2008), effective models of PLD without polycystic kidneys would be useful to address clinical and mechanistic issues. However, infertility due to this disease limits the development of animal models (Lovaglio et al., 2014). PLD-patient hiPSCs differentiated into cholangiocyte organoids can recapitulate the disease phenotype *in vitro* and could be used to identify compounds that might reduce the cyst size (Sampaziotis et al., 2015).

5.2 Tissue engineering

Cholangiopathy treatments are based on pharmacotherapies that are largely ineffective. Orthotopic liver transplantation (OLT) remains the only curative treatment for cholestatic liver diseases, representing up to 5% of patients receiving an OLT in the US each year (Khungar & Goldberg, 2016). However, the number of available transplantable organs remains insufficient to cover the needs of patients suffering from liver diseases. Many efforts are thus made to build a bioengineered bile duct to provide an alternative to liver transplantation for cholangiopathy treatments. Several approaches have been tested like the use of biomaterials to allow the regeneration of the bile duct *in vivo*, or the coculture of liver cells to build hepatobiliary organoids *in vitro*.

Aikawa et al., for example, implanted a bioabsorbable patch made of polycaprolactone and polyglycolic acid (PGA) at the site of a bile duct defect in pig to promote regeneration of the duct (Aikawa et al., 2010) with some success. A few years later, another study showed that affected extrahepatic bile duct of guinea pigs could be replaced by three-dimensional collagen duct modified with 2% agarose hydrogel (Pérez Alonso, Del Olmo Rivas, Romero, Cañizares Garcia, & Poyatos, 2013).

Because of the importance of multicellular interactions during early liver development, other studies have focused on this parameter and cocultured liver cells for tissue reconstruction. If the first report of this liver cell coculture system used primary cells (Takebe et al., 2012), other systems focused on interactions between pluripotent stem cell-derived hepatocytes and cholangiocytes. Hepatobiliary organoids present a bile duct-like morphology and are composed of both differentiated hepatocytes (expressing specific markers like albumin and α1-antitrypsin) and cholangiocytes (expressing CK7 and CFTR). These organoids can secrete several types of bile acids into the supernatant and produce albumin (Guan et al., 2017).

More recently, Wu et al. reported the formation of 3D hepatobiliary organoids differentiated from hiPSCs (Wu et al., 2019). In these 3D structures, induced hepatocytes were functional as they could take up indocyanine green, accumulate lipid and glycogen, secrete albumin and urea, and present a CYP3A4 activity. The biliary structures of the organoids could efflux rhodamine and store bile acids. Furthermore, the authors transplanted these 3D structures under the splenic capsule of NOD-SCID

immunodeficient mice. More than 8 weeks post-transplantation, biliary duct-like structures were stained positive for human CK19 and hepatic clusters were positive for human albumin, showing a potential use of these structures for *in vivo* regenerative medicine.

5.3 Regenerative medicine

As previously mentioned, surgical therapies for cholangiopathies, like orthotopic liver transplantation, are limited in scope and availability. Furthermore, there is a high risk of biliary complication when liver donation occurs after cardiac death or leaks due to difficult anastomosis in the case of living donor transplant. For these reasons, many efforts have attempted to use hiPSC-derived cholangiocytes or organoids to reconstruct impaired biliary tree *in vivo*.

In 2015, several studies reported the transplantation of iPSC-derived cells, either cholangiocytes (De Assuncao et al., 2015) or hepatoblasts (Ogawa et al., 2015), into mice. Assuncao et al. used a mouse model of impaired biliary tree by distal common bile duct clamping and dissection of the gallbladder. After injection of iPSC-derived cholangiocytes on site, they observed emerging structures expressing biliary markers and showing the regeneration potential of the approach. Ogawa et al. obtained the same results using a coculture system of hPSC-derived hepatoblasts with stromal cells.

Sampaziotis et al. reported in 2017 the development of a bioengineered biliary tissue and bile duct structures using healthy extrahepatic cholangiocytes (Sampaziotis et al., 2017). They seeded cells on collagen tubes, transplanted them into an extrahepatic biliary injury (EHBI) mouse model and showed a regeneration of the compromised bile duct in terms of architecture, structure, markers, and functions. They then used polyglycolic acid (PGA) scaffolds and transplanted the seeded scaffolds into their mouse model, highlighting the regeneration capacity of this system without any tumor formation.

6 Overview of the protocol

Here, we will describe the differentiation of cholangiocytes from hiPSCs using a protocol we established based on previously published protocols (Caron et al., 2019; Dianat et al., 2014; Sampaziotis et al., 2017). We will also describe methodologies for characterization of the 3D-cultures of derived cholangiocytes including immunofluorescence and functional test using cholyl-lysyl-fluorescein (CLF). It is important to note that the last steps of this protocol can be modified depending of the size of the desired 3D structures. As previously mentioned, other approaches using hESCs or primary cells have also been developed but we will restrict our discussion on hiPSC-derived cells. Briefly, the steps of the differentiation protocol are as follow (Fig. 2A).

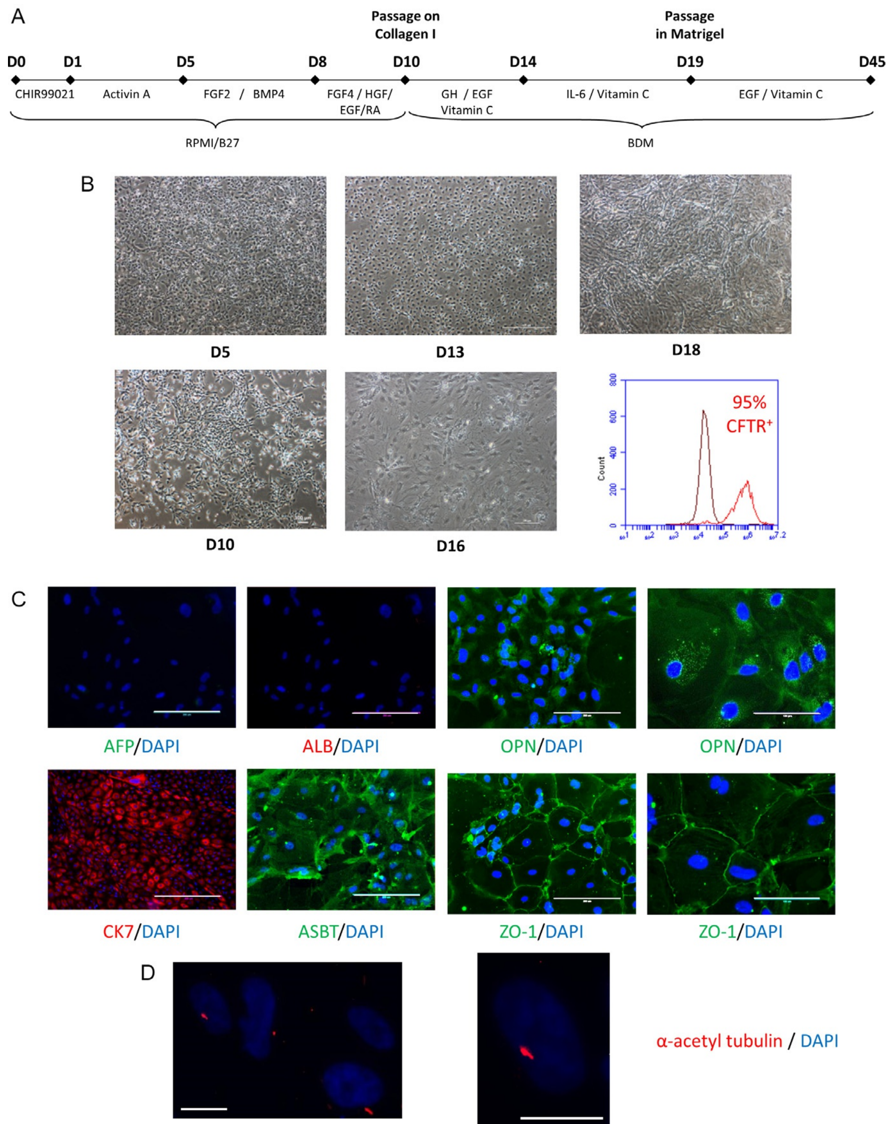

FIG. 2

Human-induced pluripotent stem cells differentiation into cholangiocytes and cholangiocyte organoids. (A) Schematic timeline of the different steps of the protocol from the hiPSC stage into cholangiocytes and cholangiocyte organoids. (B) Representative phase contrast images of the morphological changes of the cells during the cholangiocyte differentiation and flow cytometry analysis of CFTR at the end of the two-dimensional differentiation. (C) Immunofluorescence staining images showing the absence of the hepatoblast and hepatocyte markers AFP and albumin, respectively, and the expression of cholangiocyte-specific markers OPN, CK7, ASBT, ZO-1. Scale bar is 100 and 200 μm. (D) Immuno fluorescence staining analysis showing the expression of acetylated α-tubulin localized on primary cilia of cholangiocytes. Cholangiocyte nuclei were visualized by staining with DAPI (shown in blue). Scale bar is 20 μm.

1. hiPSCs are plated onto gelatin-coated plates.
2. hiPSCs are treated with 3 μM of CHIR90221 in RPMI/B27 medium for 24 h and subsequently maintained in this culture medium for 9 days in combination with different cytokine cocktails, as described in detail below.
3. At the hepatoblast stage (day 10), cells are passaged onto collagen-I coated plates in homemade Biliary Differentiation Medium (BDM) and subsequently maintained in this culture medium in combination with several cytokines like the human growth hormone, which is abundant in fetal serum and whose receptor is expressed in fetal liver when the ductal plate is formed (Simard, Manthos, Giaid, Lefèbvre, & Goodyer, 1996).
4. Cells are detached at day 19 and transferred to 3D culture conditions in Matrigel to form cholangiocyte organoids.

A step-by-step protocol is detailed in Section 7.

7 Step-by-step methods

7.1 hiPSC culture

hiPSCs are cultured in feeder-free conditions. We choose Geltrex as a matrix but Matrigel or Laminin-521 can also be used depending on the cell line used. We routinely use StemMACS™ iPS-Brew XF human as culture medium but alternative pluripotent stem cell culture media can also be used.

7.1.1 Materials and reagents

- GelTrex™ L-DEV Free hES Qualified Reduced Growth Factor (Gibco™, A1413302, Thermo Fisher)
- Complete StemMACS™ iPS-Brew XF human (130-104-368, Miltenyi Biotec)
- Penicillin/Streptomycin (Gibco™, 11548876, Thermo Fisher)
- Dulbecco's Modified Eagle Medium (DMEM)/F12 basal medium (Gibco™, 31330038, Thermo Fisher)
- Sterile Phosphate-Buffered Saline (PBS) for washing step (Gibco™, 14190250, Thermo Fisher)

7.1.2 Protocol

1. Thaw Geltrex overnight at 4 °C.
2. Add the desired volume of cold DMEM/F12 media to obtain a 2 mg/mL solution of Geltrex in a pre-chilled tube. Slowly pipet up and down to homogenate the solution. Avoid introducing air bubbles. Aliquots of this concentrated solution can be stored at −80 °C for several months.
3. When needed, thaw a Geltrex aliquot by adding the recommended cold DMEM/F12 media to obtain a 0.05 mg/mL final concentration. Add the recommended coating volume to plates.

4. Rock the plate back and forth to coat the bottom of each well. Incubate for at least 30 min at 37 °C. The plates can be sealed with Parafilm and stored at 4 °C for up to 2 weeks after coating.

7.2 Coating gelatin plates for hiPSC differentiation

hiPSC differentiation requires the pre-coating of tissue culture plates with gelatin for the cell attachment.

7.2.1 Materials and reagents

- Gelatin from porcine skin (G1890-500G, Merck)
- Sterile-filtered water (Water For Embryo Transfer, W1503, Merck)
- Dulbecco's Modified Eagle Medium (DMEM), high glucose, pyruvate (Gibco™, 11594486, Thermo Fisher)
- Fetal Bovine Serum (FBS) (Gibco™, 10500064, Thermo Fisher)
- Tissue culture plates. For the first days of differentiation we prefer 6-well tissue culture-treated plates (Corning™, 10146810, Thermo Fisher)

7.2.2 Protocol

1. Prepare 0.1% Gelatin in sterile-filtered water.
2. Add 1.5 mL of the gelatin solution per well of a 6-well plate.
3. Rock the plate back and forth to coat the bottom of each well. Incubate for at least 30 min at room temperature.
4. Aspirate the gelatin solution and add 1.5 mL of DMEM/0.1% FBS in each well. Incubate overnight at 37 °C. The plates can be sealed with Parafilm and stored at 4 °C for up to 2 weeks after overnight incubation.
5. The day after, just before seeding the cells (see Section 7.3.2), aspirate the medium and wash once with sterile PBS.

7.3 Differentiation of hiPSCs into hepatoblasts

This part of the protocol will lead to two-dimensional culture of hepatoblasts in 6-well plates. The protocol can be modified to obtain these cells in other culture plate formats.

7.3.1 Material and reagents

- Gelatin-coated culture plates (see Section 7.2 for detailed protocol).
- hiPSCs grown in feeder-free conditions. We use the A29 iPSCs reprogrammed in our laboratory using non-integrative method (Steichen et al., 2014). Cells are routinely cultured and maintained on Geltrex with weekly passage.
- Trypsin-EDTA (0.05%) (Gibco™, 11580626, Thermo Fisher)
- Dulbecco's Modified Eagle Medium (DMEM)/F12 basal medium (Gibco™, 31330038, Thermo Fisher)

- Fetal Bovine Serum (FBS) (Gibco™, 10500064, Thermo Fisher)
- RPMI/B27 medium: RPMI 1640 basal medium (Gibco™, 11534446, Thermo Fisher) supplemented with 1% L-Glutamine (Gibco™, 11500626, Thermo Fisher), 1% Penicillin/Streptomycin (Gibco™, 11548876, Thermo Fisher) and 1 × serum-free B27 supplement (Gibco™, 11530536, Thermo Fisher)
- RPMI/B27 medium w/o methionine: RPMI 1640 basal medium deprived of methionine (Gibco™, 12857552, Thermo Fisher) supplemented with 1% L-Glutamine (Gibco™, 11500626, Thermo Fisher), 1% Penicillin/Streptomycin (Gibco™, 11548876, Thermo Fisher) and 1 × serum-free B27 supplement (Gibco™, 11530536, Thermo Fisher)
- Sterile Phosphate-Buffered Saline (PBS) for washing step (Gibco™, 14190250, Thermo Fisher)
- Complete StemMACS™ iPS-Brew XF human (130-104-368, Miltenyi Biotec)
- Rho/Rock pathway inhibitor Y27632 (72307, StemCell Technologies) and CHIR99021 (04-0004, StemCell Technologies) dissolved in sterile Dimethyl Sulfoxide (DMSO) (D2438, Sigma)
- Human Activin A (130-115-011) and Fibroblast Growth Factor-basic (FGF2) (130-104-923) were purchased from Miltenyi Biotec
- LY294002 (Cayman, CAYM70920-10, VWR)
- Bone Morphogenetic Protein-4 (BMP4) (314-BP-500, R&D)
- Fibroblast Growth Factor 4 (FGF4) (100-31), Hepatocyte Growth Factor (HGF) (100-39) and Epidermal Growth Factor (EGF) (AF-100-15) were all purchased from Peprotech
- Retinoic acid (R2625, Merck)

7.3.2 Protocol

Day-2: Preparing a single-cell suspension and plating cells.

To launch the differentiation protocol, hiPSCs should be at 80–90% confluence after 48 h of culture. It is not recommended to wait more than 2 days before launching the differentiation as the cells will then start to compact which will decrease the differentiation efficacy. The optimal cell density for plating will vary depending on the cell line and testing several densities is recommended.

1. Aspirate the medium from hiPSCs and wash once with sterile PBS. Aspirate the PBS and add enough trypsin to cover the cells.
2. Incubate at 37 °C until cells detach from the plate (about 5 min).
3. Dissociate the cells by pipetting up and down and transfer the cells to a 15 mL conical centrifuge tube. Rinse the remaining cells in the plate with DMEM/0.1% FBS and add to the conical tube.
4. Centrifuge at 1000 rpm for 5 min at room temperature.
5. Carefully aspirate the supernatant and resuspend the cell pellet in StemMacs supplemented with 10 μM Y27632.
6. Seed the cells on the gelatin-coated plates prepared the day before (see Section 7.2.2).

7. The next day, aspirate the medium and add 1.5 mL of complete StemMacs in each well. It is not recommended to incubate the cells with Y27632 more than 24 h.

Day 0 at day 10:

1. Two days after plating (Day 0 of differentiation), aspirate the culture medium and add 1.5 mL of RMPI/B27 medium complemented with 3 μM CHIR99021.
2. From day 1 to day 4 change the medium daily with RPMI/B27 supplemented with 100 ng/mL Activin A, and 10 nM Ly294002. Cell mortality may occur at this time without impacting the next steps of the differentiation.
3. From day 5 to 7, change the medium daily with RPMI/B27 supplemented with 50 ng/mL Activin A, 20 ng/mL FGF2 and 10 ng/mL BMP4.
4. From day 8 to 9 of the differentiation, we use RPMI/B27 medium deprived of methionine which helps to remove remaining hiPSCs that did not engage into the differentiation process. For 2 days, supplement this medium with 30 ng/mL FGF4, 25 ng/mL HGF, 50 ng/mL EGF and 10^{-7} M retinoic acid.

At day 10, cells express specific markers of hepatoblasts, the hepatic progenitors that can then differentiate into cholangiocytes and hepatocytes (Dianat et al., 2014).

7.4 Coating collagen plates for hepatoblast differentiation into cholangiocytes

Hepatoblast differentiation into cholangiocytes requires the pre-coating of tissue culture plates with collagen-I. Instead of homemade collagen-coating culture plates, commercialized pre-coated plates (Corning™ BioCoat™ Collagen-I plates, 10033760, Thermo Fisher) can be used effectively.

7.4.1 Material and reagents

- Tissue culture plates. For the hepatoblast differentiation into cholangiocytes we prefer 12-well tissue culture-treated plates (Corning™, 10136810, Thermo Fisher)
- Collagen, type I solution from rat tail (C3867-1VL, Merck)
- Sterile Phosphate-Buffered Saline (PBS) for washing step (Gibco™, 14190250, Thermo Fisher)

7.4.2 Protocol

1. Add a defined volume of collagen I per each well of a 12-well plate for a final concentration of 5 μg collagen/cm^2. We recommend a minimum volume of 400 μL to ensure a complete covering of the well bottom.
2. Incubate 20 min at 37 °C.
3. Aspirate the collagen solution and rinse three times with sterile PBS. The plates can be sealed with Parafilm and stored at 4 °C for up to 2 weeks after coating.

7.5 Differentiation of hepatoblasts into cholangiocytes

This part of the protocol will lead to three-dimensional structures in 12-well plates. The protocol can be modified to obtain these same structures in other culture plate formats, such as 24-well plates or in LAB-TEK chamber slides.

7.5.1 Material and reagents

- Collagen-coated 12-well culture plates
- Interleukin-6 (IL-6) (130-093-932) was purchased from Miltenyi Biotec
- Epidermal Growth Factor (EGF) (AF-100-15) and human Growth Hormone (HG) (100-40) were purchased from Peprotech.
- Cell Dissociation Buffer (CDB): 0.1 mg/mL EDTA (E6758, Merck), 0.5 mg/mL Bovine Serum Albumin (BSA) (A7030, Merck) in phosphate-buffered saline (PBS) 1 ×
- Biliary Differentiation Medium (BDM): mix 1:1 of Phenol red free Williams'E culture medium (Gibco™, 10137414, Thermo Fisher): Ham's F-12 Nutrient Mix (Gibco™, 15172529, Thermo Fisher), 10^{-5} M linoleic acid-Albumin (L9530, Merck), 5×10^{-8} M 3,3′,5-Triiodo-L-thyronine sodium salt (T2752, Merck), 0.2UI Insulin (Umuline), 6×10^{-4} M human apo-transferrin (T5391, Merck), 1 mM sodium pyruvate (Gibco™, 12539059, Thermo Fisher), 1% L-Glutamine (Gibco™, 11500626, Thermo Fisher) and 1% Penicillin/Streptomycin (Gibco™, 11548876, Thermo Fisher)
- Matrigel phenol red free (Corning™, 11593620, Thermo Fisher)
- Fetal Bovine Serum (FBS) (Gibco™, 10500064, Thermo Fisher)
- Sodium taurocholate hydrate (86339-25G) and vitamin C (A4403) were purchased from Merck
- Trypsin-EDTA (0.05%) (Gibco™, 11580626, Thermo Fisher)
- Rho/Rock pathway inhibitor Y27632 (72307, StemCell Technologies)

7.5.2 Protocol

1. At day 10 of the differentiation, cells are detached from the gelatin-coated plates (see next step). Aspirate the culture medium and rinse once with sterile PBS.
2. Add 1 mL of Cell Dissociation Buffer (CDB) per well of a 6-well plate and incubate few minutes at room temperature. The incubation time depends on the cell line used but also on the cell density at this stage of the differentiation protocol and can vary from 5 to 10 min. The detachment of the cells can be monitored under a microscope as cells will become refractive under CDB action.
3. Before cell lifting, aspirate the CDB without disturbing cells. Then, detach the cells by flushing them with fresh RPMI/B27 medium. Collect the cells in a conical tube and ensure all cells have been collected by rinsing each well one additional time with fresh RPMI/B27, adding additional cells and media to the conical tube.
4. Centrifuge 5 min at room temperature at 1000 rpm. Gently aspirate the medium and resuspend the cells in 1–5 mL of Biliary Differentiation Medium (BDM)

supplemented with 6×10^{-4} M Vitamin C, 10% FBS and 1 mg/mL BSA. Use a Malassez cell counter to count the cells and adjust the medium volume to obtain a cell concentration of 7×10^{5}cells/mL. Dispatch 1 mL of the cell suspension in each well of a collagen-coated 12-well plate and incubate 4 h at 37 °C.

5. Gently aspirate the culture medium and add 1 mL of BDM supplemented with 6×10^{-4} M Vitamin C.
6. From day 11 to day 13, refresh the medium daily with BDM supplemented with 50 ng/mL human Growth Hormone (GH), 25 ng/mL EGF and 6×10^{-4} M Vitamin C.
7. From day 14 to 18, refresh the medium daily with BDM supplemented with 10 ng/mL IL-6, and 6×10^{-4} M Vitamin C.

If immunostaining or molecular biology analysis of two-dimensional differentiated cells is required, treat the cells for 2 days with 10 µM sodium taurocholate hydrate before analyses to allow cell proliferation (Fig. 2B–D).

8. To further differentiate the hiPSC-cholangiocytes in 3D-structures, detach the cells at day 19 with CDB. Incubate the 12- or 24-well plates in which the 3D structure will be formed at 37 °C.
9. Add 0.5 mL of Cell Dissociation Buffer (CDB) per well of a 12-well plate and incubate few minutes at room temperature. The incubation time depends on the cell line used but also the cell density at this stage of the differentiation and can vary from 5 to 10 min. The detachment of the cells can be monitored under a microscope as cells will become refractive under CDB action.
10. Before cell lifting, carefully aspirate the CDB and detach the cells by gently flushing them with fresh BDM medium. At this stage, do not fully dissociate the cells but rather leave visible cell clumps. This step is crucial for the last part of the differentiation as a single-cell suspension will not lead to 3D structure formation in Matrigel.
11. Collect the cells in a conical tube and rinse once all the wells to ensure a complete collection of the cell clumps.
12. Centrifuge 3 min at room temperature at 444*g*.
13. Carefully aspirate the culture medium and suspend the cells in an appropriate volume of a freshly prepared 50% (vol/vol) ice cold BDM/Matrigel mix supplemented with 20 ng/mL EGF, 10 µM Y27632 and 6×10^{-4} M Vitamin C. Cells should be plated at a density that allow the emerging of the 3D-structures at 80% confluence in 10 days. Optimal splitting ratios will depend on the cell line and the cell density at this step of the protocol but this is usually achieved by using 1:6 to 1:10 split ratio.
14. Mix the 50% BDM/Matrigel cell suspension on ice and form a Matrigel dome in each well of a pre-heated 12- or 24-well plate. To form a dome, use a 1000 µL pipette close to the bottom of the plate and start slowly pipetting 100 µL of the mix until a small droplet forms. At this step, ensure that the droplet does not touch the walls of the well.
15. Incubate 2–3 min at room temperature to allow the droplet to solidify.

16. Flip the plate upside down and incubate 30 min at 37 °C.
17. Flip back the plate and add 0.5–1 mL of BDM supplemented with 20 ng/mL EGF, 10 μM RI and 6×10^{-4} M Vitamin C depending of the culture plate format.
18. From day 20 to the end of the differentiation, replace the medium every 2 days with fresh BDM supplemented with 10 ng/mL IL-6, 6×10^{-4} M Vitamin C and 20 ng/mL EGF.

At the end of the differentiation, cells express specific markers of the cholangiocytes, and form 3D organoids like cysts or tubular branching structures (Fig. 3).

7.6 Characterization of cholangiocyte organoids

7.6.1 Immunofluorescence *(Fig. 4A and B)*

- Paraformaldehyde (PFA) 16% SOL. EM GRADE (15710, Euromedex)
- Dulbecco's Phosphate-Buffered Saline (PBS) with calcium chloride and magnesium chloride, 10 ×, for washing step (D1283-500ML, Merck)
- Permeabilization solution: 0.1% Triton-X100 (T9284, Merck) in PBS 1 × (D1283-500ML, Merck)
- Saturation solution: 5% BSA (A9647-100G, Merck) in PBS 1 × (D1283-500ML, Merck)
- Tween 20 (P2287, Merck)
- DAPI (D9542, Merck)
- Mounting medium Fluoromount-G® (0100-01, Cliniscience)

1. Aspirate the culture medium.
2. Add 1 mL of 4% PFA per well and incubate 20 min at room temperature to fix the hiPSC-cholangiocyte organoids in Matrigel.
3. Aspirate the PFA.
4. Wash twice with PBS 1 × during 10 min.
5. Incubate 30 min with Permeabilization solution.
6. Wash twice with PBS 1 × during 10 min.
7. Incubate 30 min with Saturation solution.
8. Incubate overnight the organoids at 4 °C with primary antibodies diluted in a solution of 1% BSA and 0.1% Triton X100 in PBS 1 ×. Polarity of cells in the cysts can be verified by basolateral and apical localization of β-catenin and F-actin respectively. Immunostaining presented in Fig. 4 were performed with anti-β-catenin (Santa Cruz, sc-7963 AF594), anti-cytokeratin 7 (DAKO, M701801-2) and phalloidin (binds F-Actin, Life Technologies, A12379)
9. Wash three times 45 min with PBS 1 ×—0.1% Tween 20.
10. Incubate the organoids 1 h at room temperature with secondary antibodies in a solution of 1% BSA and 0.1% Triton X100 in PBS 1 × in dark.
11. Aspirate the solution and incubate 10 min at room temperature in dark with a solution of 1% BSA, 0.1% Triton X100 and 1/10000 DAPI in PBS 1 ×.

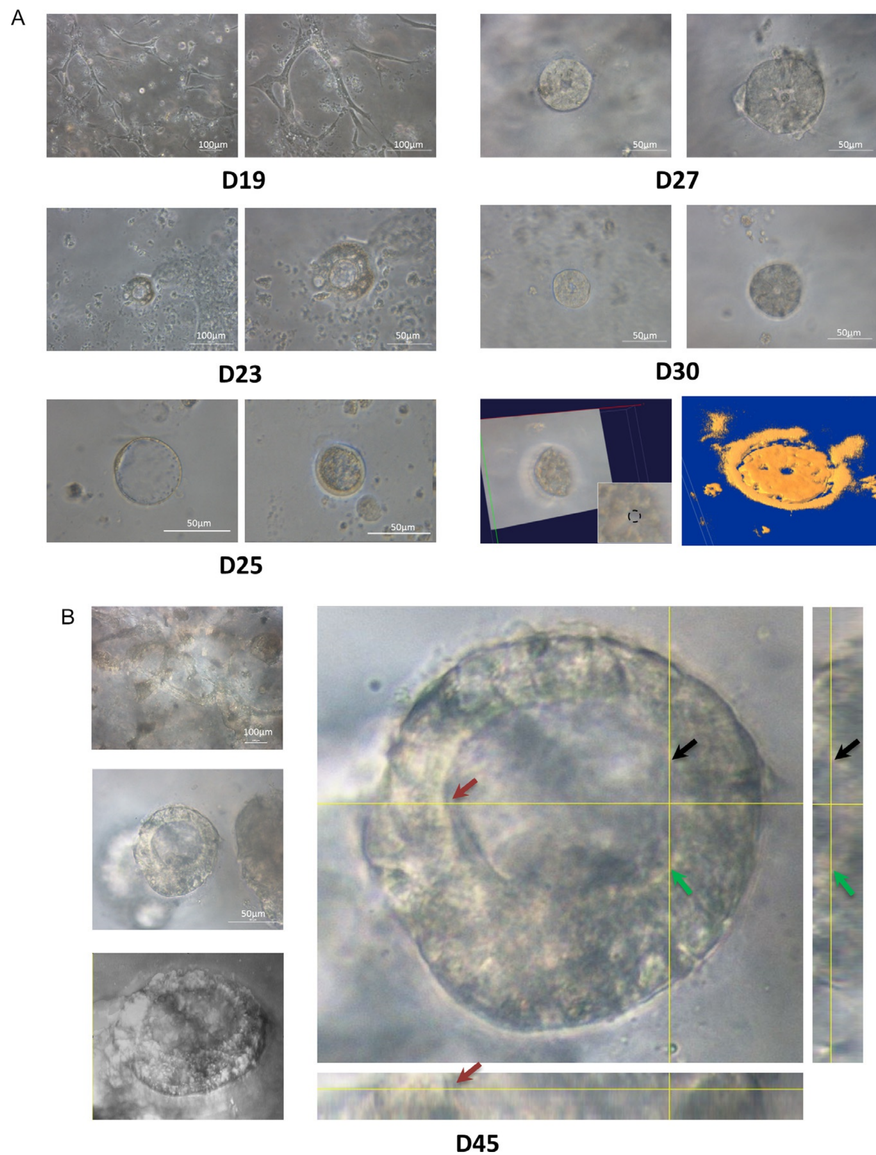

FIG. 3

Formation of hiPSC-derived cholangiocyte organoids in matrigel. (A) Phase contrast images showing the morphology of cysts and tubular-like structures in the 3D culture system and their evolution from day 19 to 45. 3D reconstruction at day 30 confirms the presence of a lumen in the cyst. (B) Cysts and tubular structures in the 3D culture system at day 45 of differentiation. Red, green and black arrows highlight the cyst lumen edges at day 45.

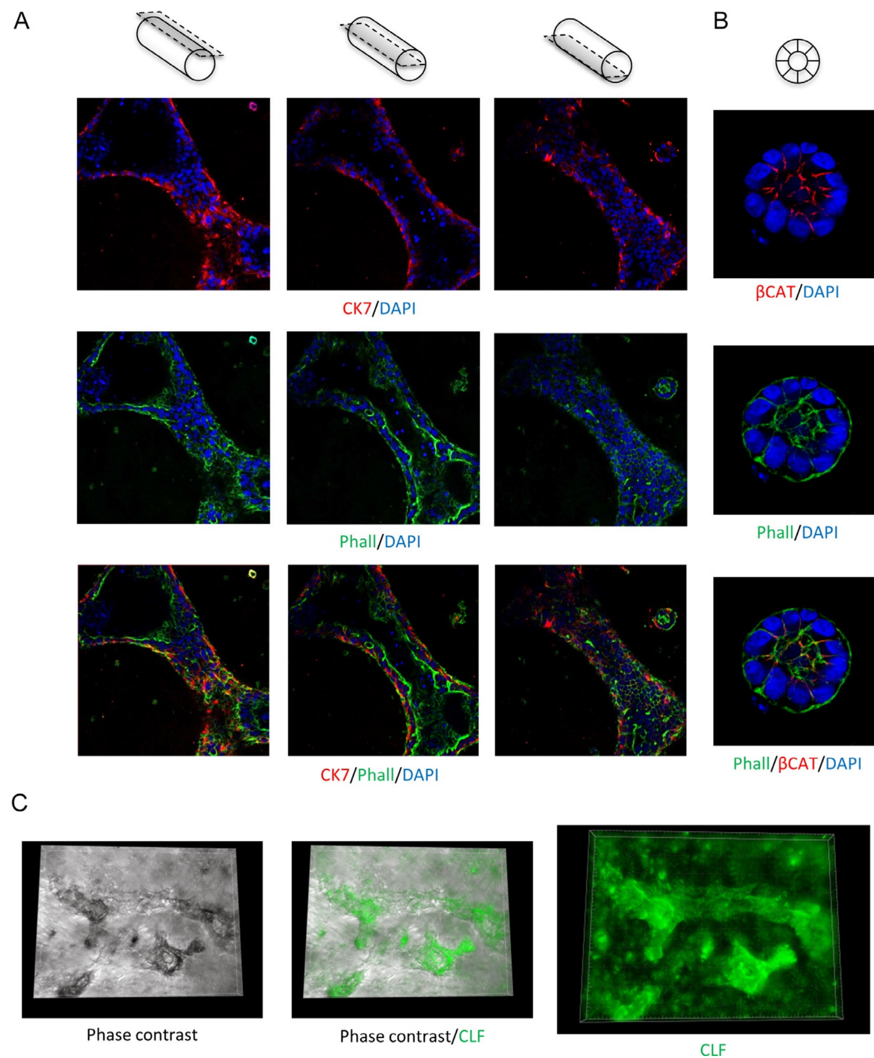

FIG. 4

hiPSC-derived cholangiocyte organoids appear in tubes and cysts lined by polarized cholangiocytes. (A) Immunofluorescence staining analysis showing expression of CK7 and F-Actin (phalloidin) in tubular structures. (B) Immunofluorescence staining analysis showing the expression of β-catenin on basolateral membrane of the cysts and of phalloidin bundles on the apical side of the cholangiocytes in the lumen. Objective x40 (C) Microscopy images and 3D reconstruction showing the active transport of cholyl-lysyl-fluorescein (CLF, fluorescent bile acid analog) in the tubular structures.

12. Wash three times 45 min with PBS 1×—0.1% Tween 20.
13. Best images can be acquired using confocal microscope. To keep the organoid staining for several days, it is recommended to use mounting medium and to cover the cells with a coverslip.

7.6.2 Functional test of bile acid transport by hiPSC-cholangiocyte organoids using cholyl-lysyl-fluorescein (CLF) (Fig. 4C)

This test allows investigating the transport of fluorescent bile salts by functional cholangiocytes using cholyl-lysyl-fluorescein (CLF) showing fluorescence accumulation inside the central lumen. For a better observation, it is recommended to use phenol red free culture medium.

- Cholyl-lysyl-fluorescein (CLF, BD-451041)
- Sterile Phosphate-Buffered Saline (PBS) for washing step (Gibco™, 14190250, Thermo Fisher)
- Phenol red free biliary differentiation medium

1. Aspirate the culture medium.
2. Wash once with sterile PBS.
3. Add 5 μM CLF and incubate 15 min at 37 °C.
4. Aspirate the CLF and wash twice with fresh phenol red free medium.
5. Visualize the fluorescence accumulation under a microscope.

8 Conclusion

Pluripotent stem cell-derived cholangiocytes and cholangiocyte organoids represent an attractive source of cells for disease modeling, drug screening, tissue engineering or regenerative medicine. The potential of 3D-culture techniques is exciting, but microscopic analysis and immunostaining must be optimized to obtain sufficiently informative images. The variability observed between organoid batches or from one cell line to another one is also to be considered and will require optimization of the protocol. However, recent advances in coculture 3D-systems are very promising and we believe that the protocol detailed in this chapter will be helpful and can be a solid basis in the establishment of more complex coculture systems.

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