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Perspective

Building consensus on definition and nomenclature of hepatic, pancreatic, and biliary organoids

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https://doi.org/10.1016/j.stem.2021.04.005

SUMMARY

Hepatic, pancreatic, and biliary (HPB) organoids are powerful tools for studying development, disease, and regeneration. As organoid research expands, the need for clear definitions and nomenclature describing these systems also grows. To facilitate scientific communication and consistent interpretation, we revisit the concept of an organoid and introduce an intuitive classification system and nomenclature for describing these 3D structures through the consensus of experts in the field. To promote the standardization and validation of HPB organoids, we propose guidelines for establishing, characterizing, and benchmarking future systems. Finally, we address some of the major challenges to the clinical application of organoids.

INTRODUCTION

Since the derivation of the first tissue-derived intestinal organoids (Ootani et al., 2009; Sato et al., 2009), immense progress has been made in the field of organoid biology, which is now an established and diverse field of research. These tissue-like 3D structures can be generated from a growing number of sources, including differentiated pluripotent stem cells (PSCs), fetal and adult primary tissue, as well as primary and metastatic tumors (Kim et al., 2020). Different types of organoids display fundamental differences in their basic characteristics, complexity, and applicability. For example, the extrahepatic bile-duct-derived organoids established by Sampaziotis et al. (2017) take the form of self-renewing epithelial luminal structures, reminiscent of bile ducts *in vivo*. In contrast, the induced pluripotent stem cells (iPSC)-derived liver bud organoids developed by Takebe et al. (2013, 2017) take the form of dense multi-cellular structures, which lack long-term self-renewal. Rather than expanding, these structures undergo mesenchymal-driven condensation to generate liver tissue with both endodermal and mesodermal components (Takebe et al., 2015). As the field of organoid biology continues to advance, there is need for consistent definitions and nomenclature to clearly describe these diverse structures. Here, we focus on organoids derived from the liver, pancreas, and biliary tree, which share a close developmental, anatomical, and physiological relationship (Ghurburrun et al., 2018).

Consistency is currently lacking when referring to tissue-derived hepatic, pancreatic, and biliary (HPB) organoids. For example, the intrahepatic bile-duct-derived organoid systems are commonly referred to as liver organoids (Huch et al., 2015), ductal organoids (Prior et al., 2019a), and cholangiocyte organoids (COs) (Hu et al., 2018). As a result, precise scientific communication is hampered, leading to a variable understanding of a given system. This makes the reproduction of results between institutions more difficult and slows scientific progress. Although renaming organoid systems can be difficult, it is essential to have a common understanding within the community. To accomplish

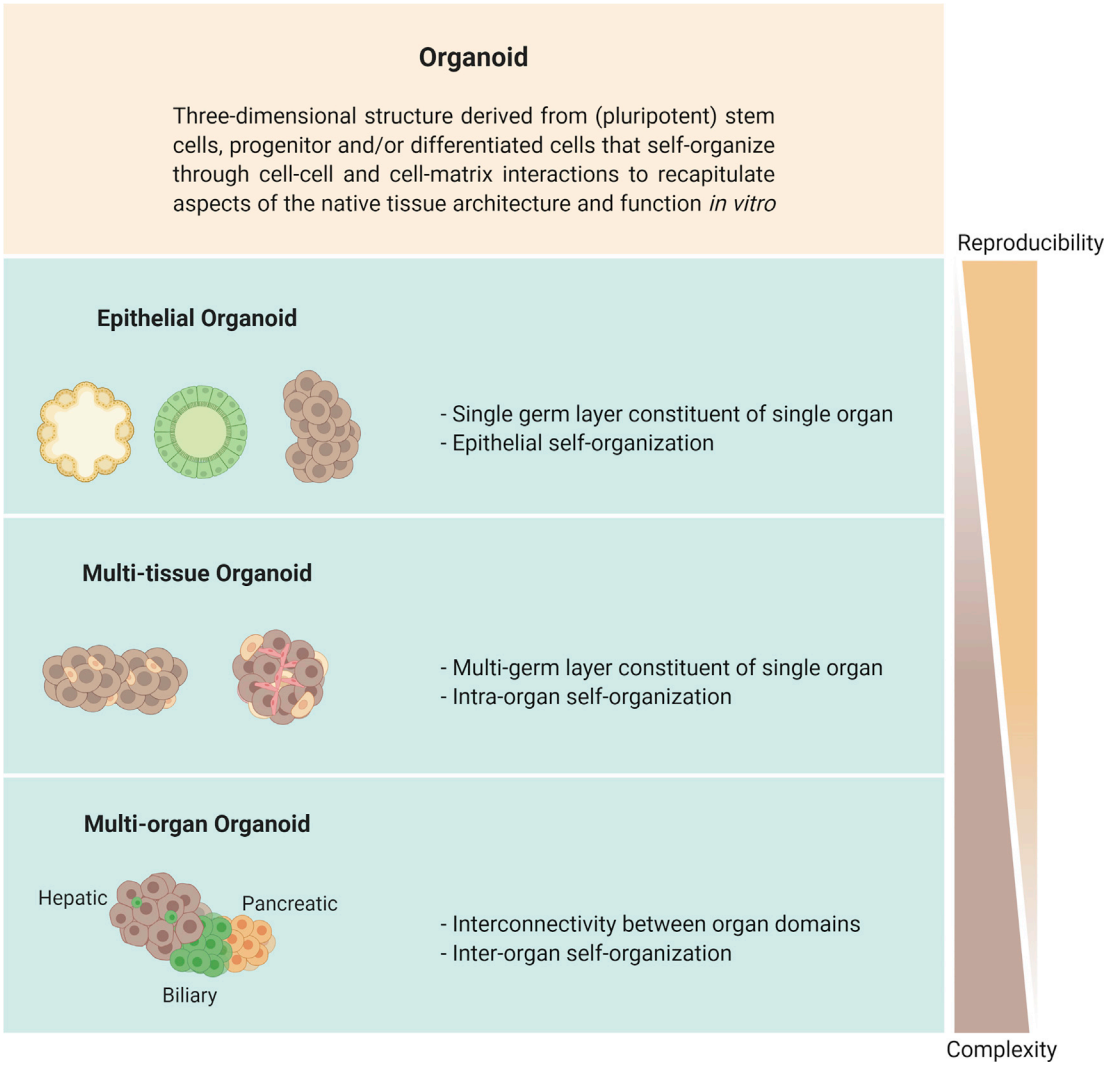

Figure 1. Overarching definition of an organoid (top panel) along with the three sub-classifications (bottom 3 panels)

this, we employed a modified Delphi method based on three successive questionnaires (Data S1: questionnaires 1–3) followed by a round-table discussion. Through the consensus of experts in the field, we propose an overarching definition of an organoid, with sub-classifications based on defining characteristics (Figure 1). In addition, we propose nomenclature for tissue-derived epithelial organoids with the goal of achieving clarity and promoting rigor in the field. Importantly, we leave room for future organoid systems that will arise as the field develops. To this end, we propose guidelines for establishing, characterizing, and benchmarking future organoids, in turn promoting the standardization and validation of organoid models. In order to provide context and background for this effort, we provide a detailed overview of the recent progress in the culture of organoids derived from the liver, pancreas, and biliary tree, as well as PSC-derived organoids differentiated toward the HPB fate. To conclude, we address some of the hurdles that need to be overcome for organoids to make their way to the clinic.

DEFINING AN ORGANOID

The concept of an organoid has been around for decades, with many scientific interpretations over the years (Simian and Bissell, 2017). However, the term's broad application, from describing small tissue explants (Shamir and Ewald, 2014) to clonally expanding cells that self-organize in 3D culture (Sato et al., 2009), has made its meaning unclear. To bring clarity to the term organoid and this growing field of research, more than 60 experts (Table S1), representing 16 countries around the world (Figure S1) have come together to define an organoid as a three-dimensional structure derived from (pluripotent) stem cells, progenitor, and/or differentiated cells that self-organize through cell-cell and cell-matrix interactions to recapitulate aspects of the native tissue architecture and function *in vitro* (Figure 1). Although early reports on the establishment of organoid systems indicated that organoids are exclusively derived from stem cells (Lancaster and Knoblich, 2014), it is now clear that organoids can also be initiated from differentiated cells,

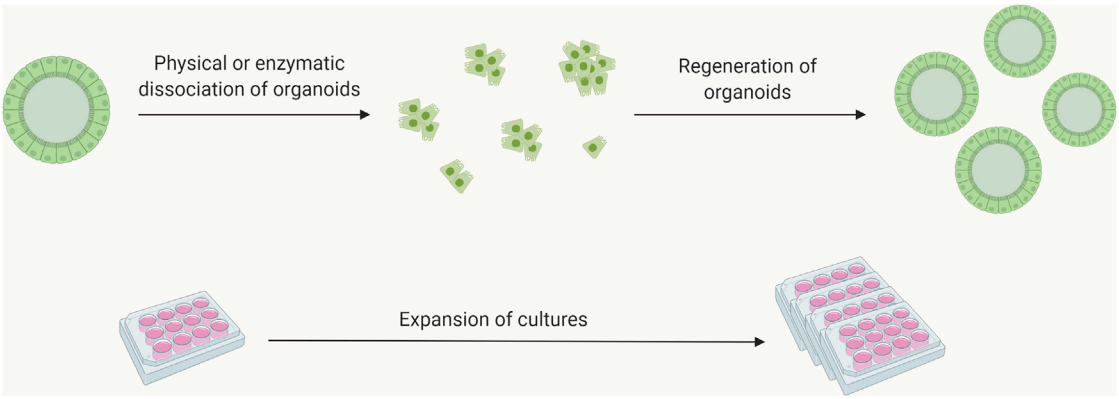

Figure 2. Self-renewal of organoids
Upon physical or enzymatic and/or chemical dissociation of organoids into fragments or single cells (followed by secondary culture in expanding conditions), cells reorganize and expand, reforming organoids.

such as cholangiocytes (Aloia et al., 2019; Sampaziotis et al., 2017). We propose that organoids can be divided into distinct groups based on defining characteristics. These include epithelial organoids, multi-tissue organoids, and multi-organ organoids (Figure 1). Of note, although we focus on HPB organoids, the proposed classification system can also be applied to organoids of other organs.

Epithelial organoids represent the most widely studied organoid type. These structures are derived from a single germ layer (endoderm, mesoderm, or ectoderm) and have the ability to self-renew under the appropriate culture conditions. In this context, self-renewal describes the repeated regeneration of organoids from organoid fragments or single cells, allowing for the serial expansion of cultures (Figure 2). Epithelial organoids exemplify this characteristic through the ability of these structures to form from the clonal expansion of a single cell (Hu et al., 2018; Huch et al., 2015; Sato et al., 2009, 2011). As epithelial organoids expand, cells polarize and specialize to reproduce aspects of the native epithelium (Sato et al., 2009). Remarkably, upon physical fragmentation or enzymatic and/or chemical dissociation of epithelial organoids into single cells or disordered cell aggregates (followed by secondary culture in expanding conditions), cells reorganize and proliferate to reform organoids (Huch et al., 2015; Sampaziotis et al., 2017). As their name implies, epithelial organoids do not harbor the mesodermal components normally present in native tissue. That withstanding, in some cases, epithelial organoids are co-cultured with supporting cells; however, these cells do not become a part of the epithelial organoid (Palikuqi et al., 2020; Wang et al., 2020). More broadly, we propose that, for cells to be constituents of an organoid, they must be functionally integrated into the overall structure and synchronized with the proliferative state of the organoid.

Multi-tissue organoids (Figure 1) are established through the co-culture of cells derived from at least two germ layers (Takebe et al., 2017) or the co-differentiation of PSCs (Ouchi et al., 2019; Shinozawa et al., 2021; Wu et al., 2019). In the contexts of the liver, pancreas, and biliary tree, multi-tissue organoids are composed of cells of endodermal and mesodermal origins. Unlike epithelial organoids, current protocols do not support the self-renewal of multi-tissue organoids, which would require the coordinated expansion of parenchymal and supporting cell types. Instead, cells interact to attain a stable level of maturity and function (Ouchi et al., 2019; Takebe et al., 2017; Wu et al., 2019). An advantage of multi-tissue organoids is their tissue-like, hetero-cellular composition. Multi-tissue organoid systems are well placed for studying the heterotypic cell-cell interactions of multiple cell types normally present in the native tissue. Importantly, these cultures show intra-organ self-organization between epithelial and supporting cell types, similar to that of the native tissue (Takebe et al., 2017; Vyas et al., 2018).

Multi-organ organoids (Figure 1) are the most complex and least described organoid type, with only one report in the context of HPB organoids (Koike et al., 2019). Characteristic to this subtype is inter-organ developmental self-organization patterns. As demonstrated by Koike et al., these systems hold great promise for the study of organogenesis, a process governed by several boundary tissue interactions (Strand et al., 2010). HPB multi-organ organoids could be maintained in culture for at least 90 days, not only displaying multiple HPB organ domains but also showing interconnectivity between them. We anticipate the emergence of additional multi-organ organoid systems in the years to come.

Is a spheroid an organoid?

The term spheroid describes 3D cell aggregates that form in the absence of a predefined culture substrate to adhere to (Fennema et al., 2013). Common techniques to generate spheroids include hanging-drop and ultra-low attachment cultures, which encourage cell-cell interaction while discouraging cell-substrate/matrix interaction (Cui et al., 2017). As a result, cells interact to form a compact sphere, although other shapes are possible (Achilli et al., 2012). This contrasts with most organoid systems, which self-organize when placed in a matrix-rich 3D environment, such as Matrigel, with which cells can interact (Hendriks et al., 2021; Huch and Koo, 2015; Tysoe et al., 2019).

That withstanding, although there is no predefined matrix with which cells can interact during the self-organization of spheroids, extracellular matrix (ECM) cues are involved in their formation. These take the form of long-chain ECM fibers containing

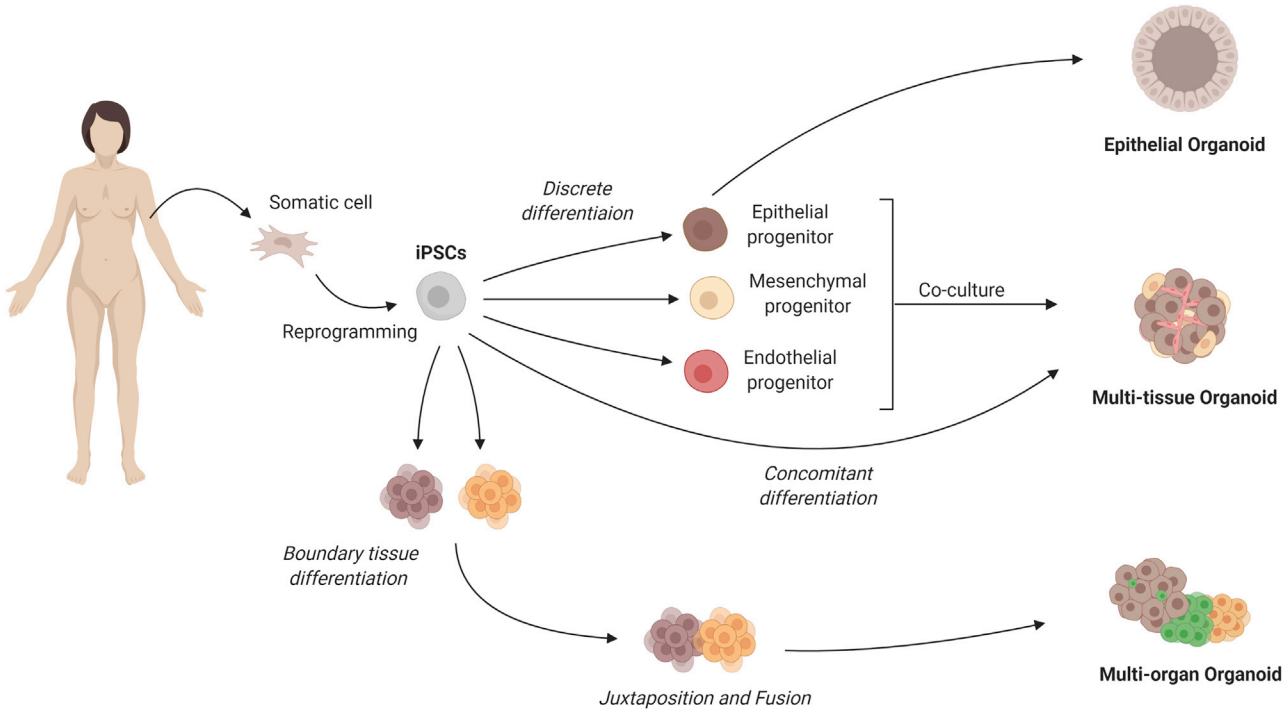

Figure 3. Generation of epithelial, multi-tissue, and multi-organ organoids from iPSCs

multiple tripeptide Arg-Gly-Asp (RGD) domains present on cell surfaces (Cui et al., 2017). Furthermore, cells secrete their own ECM molecules that likely participate in their organization. In some cases, multi-cellular spheroid systems have been shown to recapitulate architectural and functional aspects of the original tissue. For example, Bell et al. (2016) reported the generation of primary human hepatocyte spheroids with bile canaliculi and mature hepatocyte functions. The question therefore arises: when is a spheroid an organoid? Here, we propose that a spheroid is an organoid when it is composed of organ-specific cell types and satisfies the overarching definition of an organoid.

HPB ORGANOIDS DERIVED FROM PLURIPOTENT STEM CELLS

Since Yamanaka and colleagues reported the efficient reprogramming of mature somatic cells into iPSCs (Takahashi and Yamanaka, 2006; Takahashi et al., 2007), numerous protocols have been developed to direct the differentiation of iPSCs into specific cell types (Osakada et al., 2009; Spence et al., 2011; Wong et al., 2012). Although early endeavors focused on stepwise differentiation protocols exclusively in 2D (Chen et al., 2012; Hannan et al., 2013; Si-Tayeb et al., 2010; Yusa et al., 2011), there has been a shift toward the 3D culture of PSC-derived cells in recent years (McCauley and Wells, 2017). It is now possible to generate epithelial, multi-tissue, and multi-organ HPB organoids from iPSCs (Figure 3). Of note, the same techniques can also be applied to embryonic stem cells (ESCs), although their use is more limited due to ethical concerns (Lo and Parham, 2009). In the present section, we review recent progress in the culture of HPB organoids derived from PSCs.

PSC-derived hepatic organoids

One of the first steps in liver organogenesis is the development of the primordial liver bud, a structure that arises when primitive hepatic endodermal cells of the ventral foregut endoderm delaminate and invade the septum transversum mesenchyme, guided by nascent endothelial cells and the adjacent cardiac mesoderm (Ober and Lemaigre, 2018). Following these principles, Takebe et al. (2013) reported the generation of multi-tissue "liver bud" organoids from the co-culture of iPSC-derived hepatic endodermal progenitors, human umbilical vein endothelial cells (HUVECs), and mesenchymal stem cells (MSCs). In 2017, the system was reintroduced with both endodermal and mesodermal compartments being derived exclusively from iPSCs (Takebe et al., 2017). Upon transplantation into immunocompromised mice, liver bud organoids functionally interconnected with the host vasculature and engrafted. The transplanted organoids performed key hepatic functions, rescuing liver function and improving survival of mice challenged with drug-induced liver failure (Takebe et al., 2013, 2017).

In an effort to model Alagille syndrome (ALGS), a genetic disorder characterized by bile duct paucity and cholestasis, Guan et al. (2017) established a blend of morphologically diverse iPSC-derived hepatic epithelial organoids. Interestingly, organoids were predominantly composed of either hepatocyte-like cells (HLCs), cholangiocyte-like cells (CLCs), or a mixture, containing both HLCs and CLCs. Organoids were self-renewing and could be matured to perform some hepatic functions, including glycogen storage, liver-specific drug metabolism, as well as

albumin and bile secretion. Notably, organoids generated using ALGS patient-derived cells formed fewer duct-like structures and had a reduced ability to mediate biliary transport compared to controls, recapitulating the disease phenotype *in vitro* (Guan et al., 2017).

In a novel approach, Wu et al. (2019) generated hepatobiliary multi-tissue organoids from iPSCs by simultaneously inducing both endodermal and mesodermal differentiation. Their protocol promoted the co-differentiation of iPSCs to hepatic, biliary, and mesodermal lineages, evident through the CD31-marked tubular network present in the organoids (Wu et al., 2019). Similarly, Ouchi et al. (2019) developed a method to generate hepatic multi-tissue organoids through the co-differentiation of iPSCs or ESCs. Single-cell transcriptomic analysis revealed that their protocol resulted in the co-emergence of HLCs, CLCs, Kupffer-like-cells, and stellate-like-cells in the organoids (Ouchi et al., 2019). Interestingly, these systems show the concomitant differentiation of PSCs to both epithelial and mesenchymal cell types under the same culture conditions (Ouchi et al., 2019; Wu et al., 2019).

Seeking to generate highly expandable sources of hepatic endodermal organoids from PSCs, Wang et al. (2019), Akbari et al. (2019), and Mun et al. (2019) established novel protocols for the derivation and culture of self-renewing ESC- and iPSC-derived hepatic epithelial organoids. In each case, organoids expanded as epithelial cysts, morphologically resembling the bile-duct-derived organoids described by Huch et al. (2015), and could be matured to perform some hepatic functions (Akbari et al., 2019; Mun et al., 2019; Wang et al., 2019). The authors further validated their hepatic organoid models by using them to model rare genetic diseases affecting the liver, such as citrullinemia type-1 (Akbari et al., 2019), as well as hepatotoxicity and steatosis (Mun et al., 2019).

In a recent breakthrough, Ramli et al. (2020) reported the generation of PSC-derived liver epithelial organoids, containing functionally interconnected hepatic and biliary compartments. Morphologically, organoids presented a dense albumin $(ALB)^+$ hepatic core surrounded by cytokeratin $(KRT)7^+$ biliary cysts. Live imaging revealed the transport of the fluorescent compound 5 (and 6)-carboxy-2,7-dichlorofluorescein (CDF) into robust bile canaliculi networks between polarized HLCs, emptying into biliary cysts composed of CLCs. Treatment of organoids with the cholestasis-inducing drug, troglitazone, disrupted the bile canaliculi network (Ramli et al., 2020). Similarly, Shinozawa et al. (2021) reported the generation of iPSC-derived hepatic multi-tissue organoids and their use as a cholestatic-drug-screening tool. Notably, organoids could be established from cryopreserved iPSC-derived foregut progenitors, allowing for quicker derivation of cultures compared to starting with naive iPSCs (Shinozawa et al., 2021).

PSC-derived biliary organoids

In 2014, Dianat et al. (2014) described the differentiation of ESCs and iPSCs into functional CLCs. When cultured in 3D conditions, PSC-derived CLCs formed cysts morphologically resembling the cholangiocyte-derived structures reported by LaRusso and colleagues (Banales et al., 2009; Lee et al., 2008). Interestingly, when kept in culture for more than 7 days, cysts began to bud, forming branched tubular structures (Dianat et al., 2014).

In 2015, the research groups of Vallier and Ghanekar introduced novel protocols for the directed differentiation of PSCs into CLCs and their 3D culture as epithelial organoids. PSC-derived COs displayed key functions and were successfully used to model genetic diseases affecting the bile duct epithelium, such as cystic fibrosis and ALGS. Importantly, researchers were able to rescue the disease phenotypes with pharmacological intervention, validating them as a drug screening tool. The organoids were also used to study biliary development through the modulation of key pathways normally active during native bile duct development, proving to be an excellent tool, not only for disease modeling and drug screening but also for the study of the mechanisms driving bile duct development (Ogawa et al., 2015; Sampaziotis et al., 2015).

PSC-derived pancreatic organoids

In 2015, Huang et al. (2015) described the generation of pancreatic epithelial organoids from PSC-derived pancreatic progenitors. Morphologically, organoids resembled cystic structures, consisting of a single, polarized layer of epithelial cells surrounding a central lumen. Characterization of the organoids revealed that culture conditions promoted a progenitor phenotype, with low or undetectable expression of mature ductal, acinar, or islet markers. However, ductal and acinar differentiation could be promoted *in vitro* by modifying the culture conditions or *in vivo* following transplantation into immunodeficient mice. In both cases, organoids formed pancreatic exocrine structures containing carboxypeptidase A1 $(CPA1^+)$ acinar and $KRT19^+$ ductal compartments (Huang et al., 2015).

Two years later, Hohwieler et al. (2017) reported the differentiation of iPSCs and ESCs to pancreatic progenitors (PPs) and their culture as epithelial organoids. 3D culture conditions promoted the emergence of acinar and ductal lineages, which comprised 34% ± 15% and 61% ± 19% of organoid cells, respectively. Functionally, PSC-derived pancreatic organoids (POs) exhibited carbonic anhydrase activity at levels comparable to freshly isolated pancreatic ductal cells, as well as detectable levels of amylase, trypsin, and elastase activity. Global gene expression analysis revealed that PSC-derived POs clustered closely to human adult pancreatic tissue, as well as primary ductal and acinar cells. When orthotopically transplanted onto the pancreas of immunodeficient mice, organoids functionally engrafted, with signs of neovascularization and tri-lineage differentiation, including insulin-producing cells (Hohwieler et al., 2017).

More recently, Yoshihara et al. (2020) succeeded in generating human islet-like organoids (HILOs) from iPSCs. HILOs were rich in endocrine cell types, with single-cell transcriptomic analysis revealing the presence of β-, α-, and δ-cell-rich populations. When transplanted into streptozotocin (STZ)-induced diabetic non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice, HILOs quickly re-established glucose homeostasis. Furthermore, when genetically engineered to overexpress the immune checkpoint protein, programmed death-ligand1 (PDL1), HILOs could even provide glucose homeostasis in immune-competent mice. PDL1-overexpressing HILOs were shielded from immune destruction, remaining glucose responsive for 50 days. Intriguingly, HILOs treated with the interferon-γ (IFN-γ), which induces PDL1 expression in pancreatic islets,

were able to recapitulate the immune-evasive properties of transgenic PDL1-overexpressing HILOs, providing glucose homeostasis for 40 days when transplanted into immune-competent STZ-induced diabetic mice (Yoshihara et al., 2020).

With the aim of developing personalized models of pancreatic cancer, Huang et al. (2021 [this issue of *Cell Stem Cell*]) and Breunig et al. (2021 [this issue of *Cell Stem Cell*]) developed novel protocols for the generation of POs from iPSCs and their subsequent oncogenic transduction. Huang et al. (2021) generated pancreatic ductal and acinar organoids utilizing two discrete differentiation strategies. Both organoid types showed cell-type-specific function and expressed either typical ductal markers, SRY-Box transcription factor (SOX)9 and carbonic anhydrase II (CA2), or acinar markers, pancreatic transcription factor 1 and chymotrypsin C, on a protein level. When genetically engineered to express one of the cancer-associated forms of KRAS proto-oncogene, GTPase (KRAS), which bears the G12D mutation, acinar organoids exhibited ductal metaplasia *in vitro* and formed intraductal papillary mucinous neoplasm (IPMN)-like structures *in vivo* (Huang et al., 2021). In parallel, Breunig et al. (2021) demonstrated that different oncogenic mutations, alone or in combination, result in distinct morphological changes and molecular phenotypes in pancreatic ductal organoids (PDOs). For instance, a combination of oncogenic KRAS expression and cyclin-dependent kinase inhibitor 2A (CDKN2A) loss resulted in PDOs forming pancreatic ductal adenocarcinoma (PDAC)-like lesions *in vivo*, while mutant GNAS complex locus (GNAS)-expressing PDOs formed IPMN-like lesions following transplantation (Breunig et al., 2021). Taken together, these studies demonstrate the potential to model diverse cancers by engineering specific oncogenic mutations into iPSC-derived organoids.

ORGANOIDS DERIVED FROM PRIMARY TISSUE OF THE HUMAN LIVER, PANCREAS, AND BILIARY TREE

It is now possible to culture self-renewing epithelial organoids from primary tissue of the human liver (Huch et al., 2015; Hu et al., 2018), pancreas (Broutier et al., 2016; Georgakopoulos et al., 2020; Loomans et al., 2018), and extrahepatic biliary tree (Lugli et al., 2016; Sampaziotis et al., 2017; Figure 4; Table S2). To establish cultures, isolated cells or tissue fragments are embedded in a matrix-rich 3D environment, typically Engelbreth-Holm-Swarm (EHS)-based hydrogels, and supplemented with medium containing growth factors and small molecules. Within days, 3D structures begin to arise that can be serially passaged for several months (Broutier et al., 2016; Hendriks et al., 2021; Tysoe et al., 2019). Tissue-derived epithelial organoids display high levels of genetic stability and are committed to their tissue of origin, making them an attractive system, not only for *in vitro* testing but also for therapeutic applications (Prior et al., 2019a). In this section, we review the recent progress in the culture of tissue-derived HPB organoids.

Building a consensus on nomenclature of tissue-derived epithelial organoids

In building a consensus on the nomenclature of HPB organoids, we first considered the basic question: what is the most important aspect the nomenclature for organoids should reflect? For example, should it reflect the cell type of origin, the cell type of resemblance *in vitro*, or the anatomical structure the organoid most resembles? In addressing this question, the community considered the following—which aspect is most informative? In the end, consensus was reached that the nomenclature for single cell-type epithelial systems should reflect the cell type of resemblance *in vitro* and that the cell and tissue of origin should be clearly defined. In this way, you know where you begin (tissue and cell of origin)—and where you end (cell type of resemblance *in vitro*). In some cases, the two align. For example, intrahepatic cholangiocyte organoids (ICOs) and extrahepatic cholangiocyte organoids (ECOs) are derived from and resemble cholangiocytes *in vitro* (Aloia et al., 2019; Sampaziotis et al., 2017). However, for organoid systems capable of transdifferentiation, the cell type of origin is not always reflected *in vitro* (Hu et al., 2018; Wollny et al., 2016). For example, in their defined hepatocyte organoid expansion medium, the mouse hepatocyte organoids (mHOs) established by Hu et al. (2018) expand as condensed structures with typical hepatocyte morphology. However, when cultured under the conditions established by Huch et al. (2015) for the culture of ICOs , cells transdifferentiate and reorganize, expressing classical cholangiocyte markers and taking on a cystic morphology (Hu et al., 2018). Because the cell type of resemblance *in vitro* does not correspond to the cell type of origin in transdifferentiating systems, it is imperative that researchers clearly identify the cell of origin. Recent discoveries have highlighted the extensive degree of plasticity that exists between the epithelial cells of the mouse liver, with numerous reports evidencing cell fate changes between hepatocytes and cholangiocytes *in vivo* (Deng et al., 2018; Manco et al., 2019; Raven et al., 2017; Schaub et al., 2018). Whether this phenomenon also occurs in humans has yet to be unequivocally established.

For epithelial organoid systems in which multiple cell types arise, consensus was reached that the nomenclature should reflect the anatomical structure that arises. Clear examples of these systems include the intestinal organoids that arise from single leucine-rich repeat-containing G-protein coupled receptor 5 $(Lgr5)^+$ stem cells to reproduce multiple cell types of the intestinal epithelium (Sato et al., 2009) or the recently published pancreatic islet organoids that arise from single protein C receptor $(Procr)^+$ cells (Wang et al., 2020). To facilitate clear scientific communication and reproducibility, it will be important for researchers to clearly define the organoid initiating cell population(s) of multi-cell-type epithelial organoids when possible.

Intrahepatic cholangiocyte organoids (ICOs)

Since the discovery that the Wnt/β-catenin target, Lgr5, marks adult stem cells in the intestine, it was also shown to mark stem cell populations in other tissues (Leung et al., 2018; Schuijers and Clevers, 2012). This led to the hypothesis that Lgr5 may represent a bona fide marker of adult stem cells in multiple epithelial tissues (Haegebarth and Clevers, 2009). However, recent evidence suggests that, for organs with low cell turnover, such as the liver, this may not be the case (Planas-Paz et al., 2019; Sun et al., 2020). In the homeostatic liver, Lgr5 marks pericentral hepatocytes expressing the canonical Wnt/β-catenin target, Axin2 (Wang et al., 2015). Upon injury, Lgr5 and Axin2 are upregulated in hepatocytes throughout the liver (Sun et al., 2020), but not in cholangiocytes at any stage during ductular

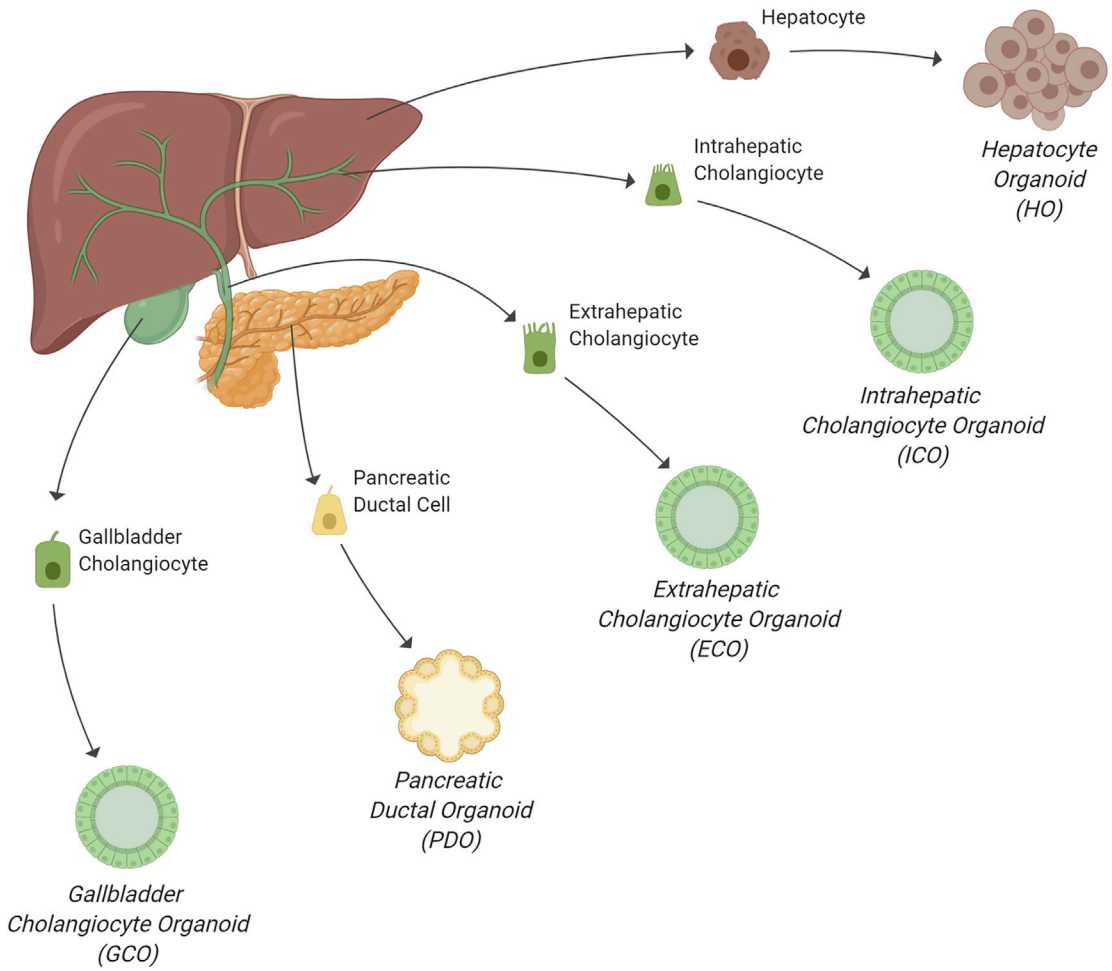

Figure 4. Nomenclature for epithelial organoids derived from primary tissue of the adult liver, pancreas, and biliary tree

reaction (Planas-Paz et al., 2019). Interestingly, in 2013, Huch et al. (2015) described a protocol for the long-term, clonal expansion of single-mouse Lgr5$^+$ liver ductal cells as cystic organoids expressing Krt19 and Krt7. Interestingly, mouse intrahepatic cholangiocytes cultured under the described conditions expressed multiple progenitor, hepatocyte, and cholangiocyte markers, suggestive of a bipotential nature. Indeed, upon modifying the culture conditions to stimulate hepatic maturation, mouse ICOs (mICOs) expressed markers of mature hepatocytes and could perform some hepatocyte functions (Huch et al., 2013a). Based on the capacity of ICOs to be differentiated to HLCs, the authors concluded that mICOs were bipotential and derived from a rare Lgr5$^+$ stem cell population of ductular origin that became activated upon carbon tetrachloride (CCl_4) injury (Huch et al., 2013a). It was subsequently demonstrated that the cells of origin were MIC1-1C3$^+$/CD133$^+$/CD26$^-$ (Dorrell et al., 2014), markers of cholangiocytes as well as biliary progenitors, leaving the question open as to whether the cell of origin was indeed a cholangiocyte or a stem/progenitor cell. Recently, Huch and colleagues employed a lineage tracing model to formally demonstrate that the organoid-initiating cells are in fact adult intrahepatic cholangiocytes that undergo Tet1-mediated epigenetic reprogramming to assume a stem/progenitor cell state, both *in vitro* and *in vivo* (Aloia et al., 2019). In support of this, Planas-Paz et al. (2019) demonstrated that Lgr5 mediated Wnt/β-catenin signaling is absent and unnecessary for Epcam$^+$ cholangiocytes to initiate a ductular reaction *in vivo*. However, upon *in vitro* culture under Wnt/β-catenin-inducing conditions, isolated Epcam$^+$ cholangiocytes form organoids and upregulate Lgr5 (Planas-Paz et al., 2019). Interestingly, Prior et al. (2019b) recently demonstrated that Lgr5 marks a sub-population of truly bipotent hepatoblasts in the early mouse embryo (embryonic day 9.5 [E9.5]; Prior et al., 2019b). Whether the ability of adult cholangiocytes to initiate organoids is restricted to a select subset of cells with increased plasticity or equally shared among biliary epithelial cells (BECs) remains to be determined.

In 2015, the culture of mICOs was adapted to support the culture of human ICOs (hICOs) (Figure 4). To do this, the medium composition was adapted to include forskolin (FSK), a cyclic AMP (cAMP) pathway agonist, and A83-01, an inhibitor of transforming growth factor β (TGF-β) receptors ALK receptor tyrosine kinase (ALK)4/5/7. Under these conditions, EpCAM$^+$ bile duct cells generated organoids with a striking efficiency of 28.4% ± 3.2%. hICOs were highly proliferative, expanding as cystic structures for several months while remaining genetically stable. Interestingly, hICOs expressed a mixture of

markers, including LGR5, the hepatocyte marker hepatocyte nuclear factor (HNF)4α, and ductal markers KRT19 and one cut homeobox (ONECUT)2. However, hICOs failed to express markers of mature hepatocytes, such as albumin or cytochrome P450 family 3 subfamily A member 4 (CYP3A4). In order to exploit the suspected bipotential character of the cells and achieve hepatocyte differentiation, culture media was developed that lacked R-spondin and FSK but included bone morphogenetic protein 7 (BMP7), fibroblast growth factor 19 (FGF19), dexamethasone, and the Notch inhibitor, DAPT. In this hepatocyte differentiation medium, hICOs upregulated several hepatocyte markers. Similar to their murine counterparts, hICOs cultured in differentiation medium acquired mature hepatocyte functions, such as albumin and bile acid secretion, glycogen storage, phase I and II drug metabolism, and ammonia detoxification. When transplanted into immunocompromised mice, hICOs could engraft and mature into hepatocytes *in vivo*, although their engraftment efficiency was low (<0.1%; Huch et al., 2015). Of note, recent single-cell analysis of the human liver has revealed extensive heterogeneity within the biliary compartment, including subsets of cholangiocytes that express classical hepatocyte markers, such as *ALB*, *SERPINA1*, and *CYP3A4* (although significantly lower compared to hepatocytes; Aizarani et al., 2019). This raises the question: does the hepatocyte differentiation protocol described by Huch and colleagues generate hepatocytes *in vitro* or are these cells simply cholangiocytes that upregulate some hepatocyte markers and perform certain functions typically attributed to hepatocytes?

Gallbladder cholangiocyte organoids (GCOs)

The protocol established to support the culture of ICOs was quickly applied to other regions of the biliary tree. In 2016, Lugli et al. (2016) demonstrated that morphologically indistinguishable organoids could be established from fragments of gallbladder and extrahepatic bile duct tissue, although they did not provide a detailed characterization of human GCOs (hGCOs) (Figure 4). Mouse GCOs expressed the Wnt target, Lgr5, as well as biliary makers Cldn3, EpCAM, Prom1, Sox9, and Itga6. Of note, Prom1 and Sox9 have been attributed to both stem cells and mature cholangiocytes. To confirm the origin of the organoids, expression of 413 gallbladder-specific and 190 liver-specific genes were analyzed. mGCOs expressed the gallbladder, but not the liver-specific genes, and had gene expression profiles that distinguished them from mICOs (Lugli et al., 2016).

In a recent breakthrough, Sampaziotis et al. (2021) demonstrated that hGCOs could be used to regenerate the intrahepatic bile ducts of the human liver. To accomplish this, the bile ducts of human livers receiving normothermic machine perfusion were infused with GCOs expanded under the conditions described for the culture of ECOs (see next section). Histological analysis of recipient livers revealed that transplanted organoids engrafted in the intrahepatic biliary tree, regenerating ~40%–85% of the injected ducts. Furthermore, livers transplanted with GCOs had no evidence of cholangiopathy, although control livers not receiving cells demonstrated evidence of ischemic injury and loss of epithelial continuity (Sampaziotis et al., 2021).

Extrahepatic cholangiocyte organoids (ECOs)

In 2017, Sampaziotis et al. (2017) reported the culture of human cholangiocytes derived from the extrahepatic bile ducts as self-renewing organoids. These cells could be isolated by several methods, including brushing of the common bile duct (CBD) during an endoscopic retrograde cholangiopancreatography, a minimally invasive procedure. To initiate ECOs (Figure 4), isolated cholangiocytes were embedded in Matrigel and cultured in medium supplemented with epidermal growth factor (EGF), R-spondin, and Dickkopf-related protein 1 (DKK-1). These culture conditions are in contrast to those established for the culture of ICOs, including both a canonical Wnt agonist (R-spondin) and inhibitor (DKK-1). Morphologically, the cells in ECOs had ultrastructural features characteristic of cholangiocytes, including cilia, microvilli, and tight junctions. Functional analysis of ECOs revealed that they retained key cholangiocyte functions, such as transport through multidrug resistance protein-1 (MDR1), luminal extrusion of bile acids, alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT) activity, and responsiveness to secretin and somatostatin (Sampaziotis et al., 2017).

To probe the clinical potential of ECOs, the group investigated their potential as a cell source for biliary reconstruction. To do this, ECOs were seeded onto polyglycolic acid (PGA) scaffolds and grown until confluence. After successful colonization, the ECO-seeded scaffolds were transplanted into mouse models of CBD and gallbladder injury. Notably, mice transplanted with the resulting tissue constructs survived for up to 104 days without complications. *In vivo*, scaffolds were successfully remodeled into functional biliary tissue expressing biliary markers KRT19, KRT7, HNF-1β, SOX9, and cystic fibrosis transmembrane conductance regulator (CFTR) (Sampaziotis et al., 2017). More recently, it was demonstrated that ECOs could also be used to regenerate the intrahepatic bile ducts of immunodeficient mice with drug-induced cholangiopathy. Control mice not receiving ECOs died within 3 weeks, although mice receiving ECOs survived up to 3 months with resolution of cholangiopathy and normal serum biochemistry (Sampaziotis et al., 2021).

Regional diversity of COs

In an attempt to better understand the differences between COs derived from different regions of the biliary tree, Rimland et al. (2021) established ICOs, ECOs, and GCOs from the intrahepatic bile ducts, common bile duct, and gallbladder, respectively. Importantly, each organoid type was derived and cultured under Wnt/β-catenin stimulating conditions, allowing for a close comparison. Interestingly, gene and protein expression analyses revealed that ICOs, ECOs, and GCOs are remarkably similar, despite some regional differences corresponding to the anatomical location from which cells are isolated (Rimland et al., 2021). In parallel, Verstegen et al. (2020) performed comparative analysis of ICOs and ECOs cultured under the conditions established by Huch et al. (2015) for the culture of ICOs. Under these conditions, ICOs and ECOs were highly similar, confirming the findings reported by Rimland and colleagues (Verstegen et al., 2020). It was subsequently demonstrated that bile composition is a large determinant of the regional identity of cholangiocytes located along the biliary tree. Treatment of ICOs, ECOs, and GCOs with gallbladder bile caused the cells to acquire overlapping gene expression

profiles, providing evidence for a niche-dependent model of regional cholangiocyte identity (Sampaziotis et al., 2021).

Despite these similarities, only ICOs demonstrated the ability to upregulate hepatocyte markers upon *in vitro* differentiation (Rimland et al., 2021; Verstegen et al., 2020). From an embryological perspective, the selective capacity of ICOs to upregulate hepatocyte markers makes sense. Intrahepatic cholangiocytes arise from bipotential hepatoblasts, although extrahepatic cholangiocytes arise from the caudal portion of the hepatic diverticulum (Spence et al., 2009). This could imply that ICOs, but not ECOs/GCOs, have the epigenetic landscape to support hepatocyte (trans)differentiation. Conversely, Sampaziotis et al. (2021) demonstrated that ECOs and GCOs are interchangeable with ICOs regarding their ability to regenerate the biliary epithelium. When transplanted into the mouse and human liver, ECOs and GCOs engrafted into the intrahepatic bile ducts, regenerating the biliary epithelium and resolving cholestasis. Notably, engrafted cells lost their extrahepatic gene expression signature and acquired an intrahepatic expression profile. Furthermore, the expression of other hepatic lineage markers was never observed, providing further evidence that extrahepatic cholangiocyte plasticity may be limited to the biliary lineage (Sampaziotis et al., 2021).

Hepatocyte organoids (HOs)

In 2018, Peng et al. (2018) reported the long-term 3D culture of primary mouse hepatocytes as organoids. To establish mHOs, primary hepatocytes were embedded in Matrigel and cultured in medium containing a combination of growth factors, including the small-molecule Wnt agonist, CHIR99021, and the inflammatory cytokine, tumor necrosis factor alpha (TNF-α). The culture conditions represent a novel approach to directing the expansion of a fastidious cell type through the exploitation of pro-inflammatory signals released during regeneration *in vivo*. In the contexts of liver regeneration, TNF-α activates a series of transcription factors, including nuclear factor κB (NF-κB), Janus kinase (JAK/STAT), activator protein 1 (AP-1), and Yes-associated protein (YAP), which enhance cell proliferation (Peng et al., 2018).

Under the expansion conditions described, mHOs could be expanded for at least 8 months. Withdrawal of TNF-α resulted in deterioration of cultures. Morphologically, cells in mHOs were polygonal in shape, with larger colonies taking on the appearance of condensed rosette-like structures. Upon transcriptomic analyses, it was shown that mHOs resembled proliferating hepatocytes after partial hepatectomy (PHx).

In order to mature mHOs, the group considered the functional heterogeneity of hepatocytes along the sinusoidal axis. Periportal (PP) hepatocytes (zone 1) are specialized in β-oxidation and gluconeogenesis, whereas pericentral (PC) hepatocytes (zone 3) are more proficient in xenobiotic detoxification, glycolysis, and lipogenesis (Schleicher et al., 2015). These metabolic differences are driven, in part, by the Wnt gradient generated by central vein endothelial cells (Kolbe et al., 2019). In mHOs, PP genes were strongly downregulated, so the group devised two different culture media: one *with* Wnt/β-catenin activation and one *without* Wnt/β-catenin activation to separately induce the expression of either PC or PP genes, allowing for a zonally defined maturation of mHOs. Functionally, mHOs in both differentiation media (DM) secreted albumin, with the highest levels in the PP-induction medium. Moreover, in both DM, mHOs were functional in low-density-lipoprotein uptake, canicular transport, and glycogen storage. Upon transplantation into fumarylacetoacetate hydrolase (Fah)$^{-/-}$ mice, mHOs engrafted and repopulated up to 80% of the liver parenchyma. Notably, cells spontaneously established zonated expression profiles, depending on their spatial engraftment along the porto-central axis (Peng et al., 2018).

In parallel, Hu et al. (2018) developed a similar protocol for the long-term culture of mouse hepatocytes and human fetal liver cells as organoids. However, rather than inflammatory cytokine-mediated expansion, the group utilized a medium with additional growth factors and the Wnt agonist, R-spondin. The cells comprising mHOs were of typical hepatocyte morphology, as revealed by transmission electron microscopy. Immunofluorescence staining showed that mHOs had strong Alb expression and were negative for the cholangiocyte markers keratin (K)19 and K7. In expansion conditions, mHOs resembled hepatocytes after PHx, expressing a combination of mature hepatocyte markers, cell-cycle markers, and proliferation markers, as well as the fetal (immature) hepatocyte marker alpha fetoprotein (Afp) (Hu et al., 2018). Similarly, Prior et al. (2019b) showed that single embryonic mouse Lgr5^{+} bipotent hepatoblasts could be expanded *in vitro* and fated to either the cholangiocyte or hepatocyte lineage, depending on the culture conditions. When cultured in hICO expansion medium, hepatoblasts would form COs. However, when cultured in the hepatocyte medium for expanding human hepatocytes in 2D (Zhang et al., 2018), cells would form mHOs, which secreted high levels of albumin while retaining their embryonic nature (by expression of Afp; Prior et al., 2019b).

Building on their murine work, the protocol for the culture of mHOs was adapted to support the long-term culture of human fetal liver cells as organoids, though the cellular origin of the organoids was never demonstrated. As the plating efficiency was low (<1%), it is possible that a rare stem/progenitor cell is the cell of origin. Fetal-liver-derived organoids were distinct from COs and morphologically resembled mHOs. Transmission electron microscopy revealed typical hepatocyte features, including nuclei with prominent nucleoli with fibrillary centers and decondensed chromatin, large numbers of mitochondria, tight junctions, and autophagic vacuoles. Remarkably, a network of bile canaliculi leading to small lumens was observed within organoids, indicating that cells were not only polarized but also interconnected. Similar to mHOs, bulk transcriptomic and functional analysis of fetal-liver-derived organoids were more comparable to primary human hepatocytes than to cholangiocytes (Hu et al., 2018).

The authors also established organoids morphologically resembling fetal-liver-derived organoids from pediatric and adult livers; however, their expansion potential was limited (<2.5 months). Importantly, organoids from both fetal and pediatric donors were capable of repopulating the hepatocyte compartment of Fah$^{-/-}$ mice to a significant extent, demonstrating that, regardless of the cell of origin, cells could complete their differentiation/maturation into hepatocytes *in vivo*. Notably, pediatric and adult-liver-derived organoids showed higher engraftment than fetal-liver-derived organoids, providing additional evidence that transplant success can be positively correlated with the maturation status

of the cell (Hu et al., 2018). Taken together, these studies represent a breakthrough in the long-term culture of primary hepatocytes, a historically fastidious cell type. However, there is need for further improvements in culture conditions in order to support the long-term expansion of adult human hepatocytes as organoids (Figure 4).

Pancreatic organoids (POs)

In 2013, the group of Grapin-Botton demonstrated that embryonic mouse pancreatic progenitors could be cultured as self-organizing 3D structures with tri-lineage (acinar, ductal, and endocrine) differentiation potential. Depending on the culture conditions, organoids could be expanded as hollow spheres or induced to form more complex branching structures that did not allow for passage (Greggio et al., 2013). The Clevers laboratory went on to establish long-term, self-renewing adult mouse pancreatic ductal organoids (mPDOs) using a protocol similar to that for the culture of mICOs. Under these conditions, mPDOs could be expanded as cystic structures that lacked an endocrine compartment. However, following transplantation, endocrine differentiation was stimulated (Huch et al., 2013b).

In 2015, the culture of mPDOs was adapted to support the expansion of adult human pancreatic ductal organoids (hPDOs) (Figure 4; Boj et al., 2015). Concurrently, Bonfanti et al. (2015) demonstrated that the 3D culture conditions established for the expansion of adult mPDOs could also support the expansion of fetal mouse and human pancreatic progenitors as organoids. As in previous cases, manipulation of the culture conditions could promote endocrine differentiation, which was shown to be negatively regulated by EGF signaling (Bonfanti et al., 2015).

Since their establishment, culture conditions for hPDOs have improved. With the removal of Wnt ligands and the addition of prostaglandin E2 and FSK, hPDOs could be mass expanded long term (Georgakopoulos et al., 2020). Under these conditions, hPDOs resembled the primary tissue closely on the gene expression level, except for Wnt target and progenitor markers, which were significantly upregulated *in vitro*. When initiated from single pancreatic ductal cells, hPDOs resembled hollow spheres, with occasional budding structures along the periphery of a central lumen composed of a polarized monolayer of epithelial cells (Georgakopoulos et al., 2020). As in the case of cholangiocytes, whether the ability of pancreatic ductal cells to initiate organoids is restricted to a discrete subset of cells or equally shared among pancreatic ductal cells has yet to be determined. Single-cell RNA sequencing (scRNA-seq) has revealed heterogeneity between ductal cells of the pancreas, though the implications for *ex vivo* culture and differentiation potential were not explored (Baron et al., 2016).

Acinar cells (the other cell type of the exocrine system) have also been cultured as organoids under conditions similar to those for the culture of PDOs. However, upon *in vitro* expansion of single acinar cells, cells gradually lost their acinar-cell expression and transdifferentiated into PDOs (Wollny et al., 2016).

When complete tissue biopsies are used for organoid initiation, larger "budding" structures have also been described (Loomans et al., 2018). In these structures, aldehyde-dehydrogenase-expressing cells localized in the tips of the budding structures displayed progenitor characteristics and could be partially differentiated toward the endocrine fate. Although no successful mature cells from the endocrine lineage could be established *in vitro*, transplantation of differentiated cells showed formation of ~1% insulin-positive cells *in vivo* (Loomans et al., 2018).

More recently, mouse pancreatic islet organoids were established from newly discovered Procr$^+$ endocrine precursor cells. When sorted and co-cultured with endothelial cells, this Procr$^+$ population could successfully form organoids under the influence of a medium containing EGF, FGF2, heparin, and vascular endothelial growth factor α. When analyzed by scRNA-seq, it was demonstrated that, although the majority of cells present were β cells, all the cell types of the pancreatic islet were present in the organoid and could successfully be used to temporarily cure diabetes in mice (Wang et al., 2020).

ORGANOIDS DERIVED FROM PRIMARY AND METASTATIC TUMORS OF THE LIVER, PANCREAS, AND BILIARY TREE

Definition and nomenclature for organoids derived from tumors

Following the discovery that tissue-derived epithelial cells could be cultured as self-renewing organoids, it was demonstrated that neoplastic epithelial cells derived from primary and metastatic tumors of the liver (Broutier et al., 2017; Nuciforo et al., 2018), pancreas (Boj et al., 2015), and extrahepatic biliary tree (Saito et al., 2019) could also be cultured as self-renewing 3D structures. Similar to non-tumor epithelial organoids, organoids derived from tumors self-organize through cell-cell and cell-matrix interactions. However, rather than recapitulating aspects of the healthy tissue, organoids derived from tumors capture the histological organization of the native tumor, and retain the genomic landscape, gene expression profile, and tumorigenic potential of the original tumor, providing a novel tool to study cancer *in vitro* (Broutier et al., 2017). In literature, consistent nomenclature for these systems is lacking and they are commonly referred to as tumor organoids, canceroids, or tumoroids (Kim et al., 2020; Porter et al., 2020). To distinguish organoids derived from tumors from other 3D cancer cell models, consensus was reached on naming these systems tumor organoids. Furthermore, it was decided that the nomenclature for tumor organoid systems should reflect the nomenclature of the associated tumor (Figure 5; Table S3). In this section, we review the recent progress in the culture of tumor organoids derived from the liver, pancreas, and biliary tree. For details on the applications of tumor organoids, the reader is directed elsewhere (Lau et al., 2020; Lo et al., 2020).

Tumor organoids derived from hepatocellular carcinoma

Malignant tumors of hepatocyte origin are referred to as hepatocellular carcinomas (HCCs). Similar to hepatocytes, culturing neoplastic cells from HCCs is difficult, with the culture of well-differentiated HCCs (<5% proliferative cells) still elusive. However, poorly to moderately differentiated HCCs are amenable to *in vitro* culture as hepatocellular carcinoma organoids (HCCOs) (Figure 5), as demonstrated by the Huch laboratory (Broutier et al., 2017). Morphologically, HCCOs resembled dense spheroidal structures and typical HCC markers, such

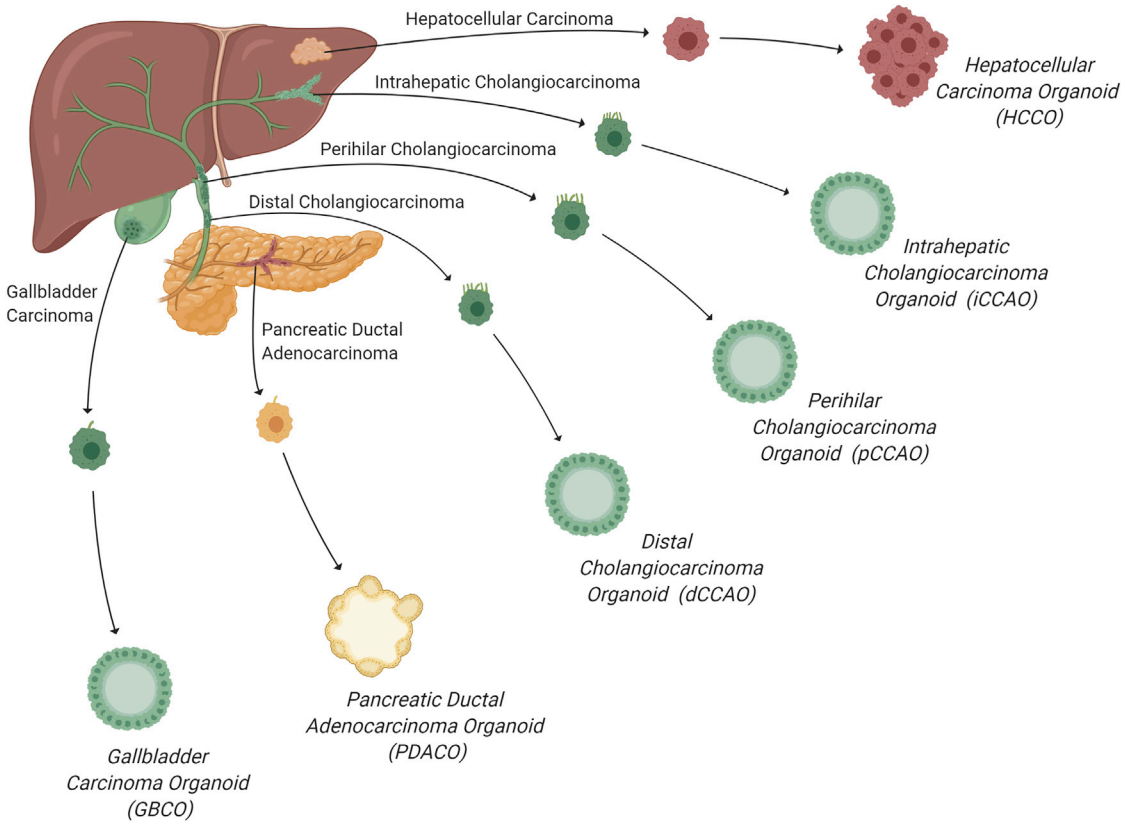

Figure 5. Nomenclature for tumor organoids derived from primary or metastatic tumors of the liver, pancreas, and biliary tree

as AFP, were expressed on a protein level. Bulk RNA sequencing revealed high correlation between original tumor samples and the resulting organoid cultures. In-depth analysis of tumor-specific mutations revealed organoids presented the same mutations as the original tumor. Importantly, it was shown that, upon subcutaneous transplantation of HCCOs, novel tumors with similar histopathological characteristics were formed, demonstrating the HCCOs retain their oncogenic potential after *ex vivo* culture. It is important to note that the culture conditions also supported the outgrowth of cells from combined hepatocellular-cholangiocarcinoma (cHCC-CCA) tumors as organoids (cHCC-CCAO) (Broutier et al., 2017). This is a rare tumor consisting of both hepatocellular and CCA characteristics, which upon radiological examination often show features of HCC. However, upon pathological examination, these tumors show characteristics of both HCC and CCA (Fowler et al., 2013), highlighting the need for tumor characterization prior to culture. A subsequent study by Nuciforo et al. (2018) demonstrated that similar organoids could be cultured from needle biopsies of the tumor, making personalized organoid models of HCCs available to a broader patient population. Because only a minority of patients would receive a surgical resection, and because patients receiving resection are less likely to receive systemic therapies (Forner et al., 2018), tumor-needle biopsies represent a convenient tissue source. More recently, hepatoblastoma organoids were established under the culture conditions described for the culture of HCCOs (Saltsman et al., 2020).

Tumor organoids derived from CCA

CCAs represent a heterogeneous group of malignancies that can be divided according to their anatomical location (intrahepatic, perihilar, and distal). Different subtypes of CCAs display distinct pathological features, prognosis, and therapeutic options (Banales et al., 2020). As CCA organoid systems are established, it is important to specify the anatomical region of the biliary tree from which the organoids are derived.

In 2017, Huch and colleagues demonstrated the feasibility of culturing neoplastic epithelial cells from intrahepatic CCAs (iCCAs) as organoids (Figure 5). Intrahepatic CCA organoids (iCCAOs) could be established from both poorly and well-differentiated tumors. Gene expression analysis revealed that iCCAOs resembled the native tumor *in vitro*. Protein-expression analysis revealed the presence of typical iCCA markers, including KRT7 and KRT19, although HCC markers, such as albumin and AFP, were absent. Similar to HCCOs, iCCAOs retained the mutational landscape of the original tumor. When subcutaneously transplanted in mice, iCCAOs formed tumors with 100% efficiency (37/37 attempts; n = 2 CCA lines), taking on glandular structures similar to primary iCCAs (Broutier et al., 2017). In parallel, Lampis et al. (2018) established iCCAOs from a patient with a highly chemo-refractory iCCA. *In vitro*, iCCAOs maintained their chemo-resistant nature, allowing researchers to investigate the possible mechanisms of chemo-resistance *in vivo* (Lampis et al., 2018).

Building on this work, Saito et al. (2019) established tumor organoids from other regions of the biliary tree and pancreas.

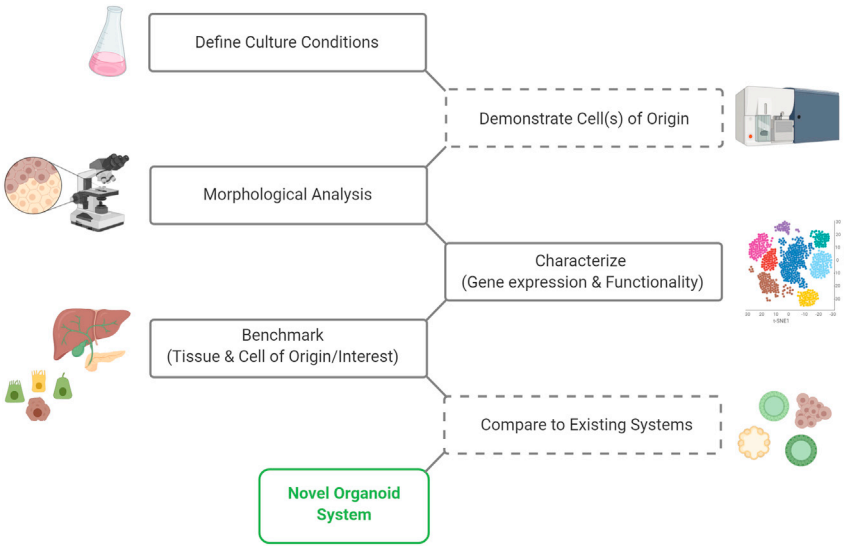

Figure 6. Guidelines for the establishment of novel organoid systems
Dotted boxes represent facultative steps.

These included gallbladder carcinoma organoids (GBCOs) (Figure 5), pancreatic ductal adenocarcinoma organoids (PDACOs) (Figure 5), and neuro-endocrine carcinoma (NEC) organoids of the ampulla of Vater. However, overall long-term success ratios were low, with only 1/3 of tumor cells growing out as organoids, similar to previous studies performed with intestinal organoids (van de Wetering et al., 2015). Notably, tumor organoids were sensitive to specific drug therapy compounds depending on the underlying mutation(s) (Saito et al., 2019).

Tumor organoids derived from pancreatic tumors

In 2015, the groups of Clevers and Tuveson demonstrated the feasibility of culturing tumorous lesions, both primary and metastatic, as tumor organoids (Boj et al., 2015). Unfortunately, most PDACs are diagnosed late. As a result, many patients diagnosed with a PDAC do not undergo surgical resection due to the advanced stage of the disease (Ryan et al., 2014). To explore the cancer screening potential of PDACOs (Figure 5), the authors demonstrated the feasibility of initiating cultures from biopsies obtained during routine fine-needle aspiration (FNA). When screened for common malignant mutations, tumor organoids were found to have mutations similar to the primary tumors from which they were derived. Upon transplantation into mice, healthy control organoids formed ductal structures with low efficacy (~9%), whereas PDACOs formed intraductal neoplasm-like lesions with high efficacy (75%). Over time, the lesions progressed and formed invasive metastatic tumors, demonstrating the value of these organoids for modeling disease progression.

More recently, Seino et al. (2018) revealed the existence of three separate tumor subtypes in 39 PDAC patients, based on Wnt and R-spondin niche factor dependencies. Tumor organoids could be established from surgical resections, FNA, and ascites (liquid) biopsies. PDACOs resembled the original tumor, presenting tumor-specific mutations, and maintained the ability to form tumors *in vivo* after *ex vivo* culture.

STANDARDIZATION AND VALIDATION OF HPB ORGANOIDS

Numerous reports describe contrasting methods of generating HPB epithelial organoids from the same cell source (Broutier et al., 2016; Lugli et al., 2016; Rimland et al., 2021; Tysoe et al., 2019). As a result, the variability of a given system increases. This makes drawing comparisons between studies more difficult, hampering translation of research and calling into question the validity of each system. To further complicate the matter, organoid complexity is increasing at a rapid pace. For example, Nikolaev et al. (2020) recently reported the generation of perfusable mini-gut and bile duct organoids on a chip by applying tissue engineering approaches to build scaffolds within preformed hydrogel networks. As novel organoid systems are developed, it is essential to maintain the clarity the present effort has sought to bring. To facilitate this, we propose guidelines (Figure 6) for researchers to follow when establishing a novel system or refining an existing one.

First, researchers should clearly define reproducible culture conditions. When possible, we encourage the use of recombinant growth factors or small molecules rather than conditioned medium in the culture of organoids. Authors should then strive to demonstrate the suspected cell(s) of origin. In animal systems, such as the mouse, this can be done through lineage tracing, but for human systems, an alternative method, such as fluorescence-activated cell sorting, could be employed. When this is not possible due to a lack of defined markers with which to sort cells, the tissue of origin should be clearly delineated. Next, the organoid system should be characterized on a morphological, gene expression, and functional level, both before and after long-term culture. When performing morphological analysis, a combination of microscopy techniques should be employed, such as light, confocal, and electron microscopy. Together, these imaging modalities will allow for a detailed analysis, not only of the organoids' overall structure but also of individual cells and their histoarchitecture (which should be compared to the primary cells/tissue the organoid resembles *in vitro*). When possible, we encourage the use of emerging technologies, such as single-cell analysis, for organoid characterization. When conducting these analyses, the organoid system should be benchmarked against the tissue and cell of origin/interest and, ideally, compared to previously established systems. To this end, the construction of an open-source, high-quality dataset containing single-cell data of HPB primary tissue and the corresponding organoids would be of great value. For human systems, we propose the use of the Organoid Cell Atlas (https://hca-organoid.eu), which was recently launched as a "Biological Network" within the Human Cell Atlas (Bock et al.,

2021). We believe the described guidelines will promote the standardization and validation of organoid systems, optimized to answer the research question at hand. Of note, although the guidelines are presented as a linear roadmap, the order of each step is at the researchers' discretion (Figure 6).

CLINICAL APPLICATION OF HPB ORGANOIDS (CHALLENGES AND SOLUTIONS)

Organoids hold great promise in the treatment of many intractable diseases in the form of advanced therapy medicinal products (ATMPs), whereby cells are injected or transplanted as organoid grafts (Hanna et al., 2016). However, before organoid technology can be translated from bench to bedside as a cell therapy, several challenges must be overcome. Here, we address (1) the elimination of animal-derived materials in the derivation and culture of organoids, (2) the mass expansion of organoids to clinically relevant numbers, and (3) overcoming immune rejection of organoids upon transplantation. In addition to these hurdles, organoids must meet basic current good manufacturing practices (c-GMPs) to be considered for clinical application, including strict quality control metrics. For more information on cGMP guidelines, the reader is directed elsewhere (Giancola et al., 2012).

Replacing EHS-based materials

Currently, efficient expansion of organoids requires Matrigel or basement membrane extract (BME) (Arnaoutova et al., 2012; Hughes et al., 2010). However, there are many issues concerning these materials. First and foremost, they are animal derived, being sourced from Engelbreth-Holm-Swarm (EHS) tumors in mice (Hughes et al., 2010). As a result, EHS-derived materials will struggle to meet c-GMPs guidelines, which precludes their use in the culture of cells intended for clinical applications. Furthermore, these extracts are not tissue specific, and there are significant batch-to-batch variations, making the reproduction of results more difficult. Thus, it is imperative to find a replacement for EHS-based materials in order to move organoids toward the clinic, as well as to promote the standardization and validation of organoid models.

Several efforts have been made to this end. Broguiere et al. (2018) reported the culture of ICOs in a hybrid fibrin/laminin-entactin hydrogel. Their results demonstrated that the addition of the laminin-entactin complex at a concentration of 2 mg/mL was sufficient to expand organoids at an efficiency comparable to Matrigel (Broguiere et al., 2018). Building on this, Ye et al. (2020) utilized synthetic polyisocyanopeptide hydrogels supplemented with recombinant human laminin-111, the primary constituent of Matrigel, to support the expansion of ICOs (Ye et al., 2020). In parallel, Sorrentino et al. (2020) reported not only the culture but also the derivation of ICOs in polyethylene glycol hydrogels functionalized with fibronectin and laminin-111. Intriguingly, replacement of full-length fibronectin with the minimal integrin recognition peptide RGDSPG (Arg-Gly-Asp-Ser-Pro-Gly) produced comparable results, allowing for a great reduction in costs compared to synthesizing full-length ECM proteins (Sorrentino et al., 2020). Similarly, Georgakopoulos et al. (2020) reported that dextran polymers modified with a peptide containing the RGD cell adhesion motif covalently crosslinked with hyaluronic acid supports the establishment as well as culture (up to five passages) of human pancreas organoids (Georgakopoulos et al., 2020). In another approach, Giobbe et al. (2019) demonstrated that ECM hydrogels derived from decellularized tissues could support the formation and growth of endoderm-derived human organoids.

Mass expansion

Standard organoid culture is an expensive and tedious process, requiring large amounts of materials, labor, and time. This limits the use of organoids in large-scale experiments, such as tissue engineering, which can require billions of cells to produce tissue constructs of clinical relevance. In an effort to overcome these limitations, the expansion of organoids in bioreactors is currently being explored (Ovando-Roche et al., 2018; Phelan et al., 2018; Przepiorski et al., 2018). Recently, Schneeberger et al. (2020) used commercial spinner flasks to mass expand ICOs. ICOs expanded in stirred suspension achieved an average of a 43-fold induction in 2 weeks, compared to a 6-fold increase in cell number in static cultures (Schneeberger et al., 2020). In another study, Kumar et al. (2019) used orbital shakers to increase the yield of iPSC-derived kidney organoids 3- to 4-fold compared to static controls. Of note, extended culture of iPSC-derived kidney organoids in stirred suspension caused visual signs of dysplasia, as well as the structural and functional decline of the organoids (Kumar et al., 2019). Although tissue-derived organoids grown under static conditions have been shown to be relatively genetically stable (Georgakopoulos et al., 2020; Huch et al., 2015), it will be important to carefully assess whether suspension culture of tissue-derived organoids compromises their genetic fitness.

Immune rejection

For organoids to make their way to the clinic, they must be immunocompatible. Although immunosuppressive drugs can help prevent graft rejection, there are several drawbacks and side effects associated with their use (Fan et al., 2015). To circumvent this, autologous cell sources can be applied. For example, tissue-derived organoids can be generated from patient cells isolated by minimally invasive methods and mass expanded *ex vivo* (Tysoe et al., 2019). Recently, it was demonstrated that COs could also be established from bile, circumventing the need for patient biopsies (Roos et al., 2021; Soroka et al., 2019). However, minimally invasive isolation methods are not available for each type of organoid, and in emergency situations, such as acute liver failure, there is a need for an off-the-shelf cell source, which means the cells will likely be of allogenic origins.

Fortunately, many encouraging efforts are currently being explored. In one approach, CRISPR-Cas9 gene editing was used to alter the expression of major histocompatibility complex (MHC) genes, generating immunocompatible allogenic iPSCs (Xu et al., 2019). That said, clinical application of gene editing has its own hurdles to overcome (Dai et al., 2016). In another study, Yoshihara et al. (2020) demonstrated that pulses of IFN-γ induced the expression of PDL1 in islet organoids, allowing them to avoid immune destruction when transplanted into diabetic mice with a functional immune system. PDL1-expressing organoids provided sustained blood sugar control without the

need for genetic manipulation for at least 40 days (Yoshihara et al., 2020).

CONCLUSIONS

As organoid technology continues to advance, so must our ability to clearly describe these complex 3D systems. To facilitate effective scientific communication between researchers, there is need for consistent nomenclature and precise language, enabling reproducibility and scientific progress. Here, we seek to harmonize the HPB organoid communities through the consensus of experts in the field. Together, we developed an intuitive classification system for organoids and nomenclature for referring to tissue-derived epithelial HPB organoids. Furthermore, in an effort to promote the standardization and validation of organoids, we proposed guidelines for researchers to follow when establishing a novel organoid system. We found the process of reaching consensus to be interactive and dynamic, stimulating scientific exchange and a holistic understanding of HPB organoids. We believe a similar process would help unify and advance other fields.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stem.2021.04.005.

ACKNOWLEDGMENTS

The L.J.W.v.d.L. lab is supported by the MLDS-Diagnostiek grant D16-26 of the Dutch Gastroenterology & Hepatology Fund (MLDS) and the Medical Delta Regenerative Medicine 4D program. The B.S. lab is supported by the European Union's Horizon 2020 research and innovation program under grant agreement no. 874586. The L.V. lab is funded by the ERC advanced grant New-Chol, the Cambridge University Hospitals National Institute for Health Research Biomedical Research Centre, and the core support grant from the Wellcome and Medical Research Council Cambridge Stem Cell Institute. The M.H. lab is funded by a Lise Meitner Excellence Program award from the Max Planck Society. The F.L. lab is supported by the Belgian National Fund for Scientific Research (FRS-FNRS, grants J.0105.19, J.0115.20, and T.0158.20), the Fund for Cancer Research (Belgium; grant 2018-078), and by the Fonds Joseph Maisin. For a detailed breakdown of the additional affiliations, locations, and roles of each HPB Organoid Consortium member, see Table S1.

AUTHOR CONTRIBUTIONS

A.M. and F.J.M.R. drafted the manuscript, designed the questionnaires, and contacted all experts from the HPB organoid consortium. A.M., F.J.M.R., M.M.A.V., H.G., E.d.K., F.L., S.J.F., W.C.P., M.H., T.T., L.V., H.C., L.J.W.v.d.L., and B.S. discussed questionnaire results and provided recommendations. All authors discussed the results and contributed to the final manuscript.

DECLARATION OF INTERESTS

H.G., M.H., T.T., L.V., and H.C. are inventors on several patents related to organoid technology.

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