Position Paper

Building a Consensus on Definition and Nomenclature of Human Hepatic, Biliary and Pancreatic Organoids

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Summary

Hepatic, biliary and pancreatic (HBP) organoids have proven to be powerful tools in the study of development, disease and regeneration, with applications ranging from basic research to regenerative medicine. Over the last decade, significant progress has been made in the culture of these three-dimensional (3D) structures. HBP organoids can now be generated from multiple sources of fetal and adult primary tissue, as well as from differentiated pluripotent stem cells (PSCs). In addition, HBP organoids have been established from primary and metastatic tumors of the liver, biliary tree and pancreas. As organoid research intensifies, with laboratories around the world culturing these diverse tissue-like structures, there is need for a clear definition and nomenclature to describe these systems. To facilitate scientific communication and consistent interpretation, we revisit the concept of an organoid and introduce an intuitive classification system and nomenclature for referring to these 3D structures through the *consensus* of experts in the field. We review recent progress in the culture of human hepatic, biliary and pancreatic organoids, with special attention to tissue-derived epithelial organoids. To promote the standardization and validation of HBP organoids, we propose a pipeline 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 intestinal organoid by Sato and colleagues (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 primary cells, stem/progenitor cells, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Kim *et al.*, 2020). Notably, 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.* take the form of self-renewing epithelial luminal structures, reminiscent of bile ducts *in vivo* (Sampaziotis *et al.*, 2017). In contrast, the iPSC-derived liver bud organoids developed by Takebe *et al.* take the form of dense multi-cellular structures which lack long-term self-renewal (Takebe *et al.*, 2013, 2017). 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 fields of hepatic, biliary and pancreatic (HBP) organoid biology continue to advance, there is need for consistent definitions and nomenclature to clearly describe these diverse structures. To accomplish this, we employed a modified Delphi method based on three successive questionnaires (supplementary information). 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). Importantly, we leave room for future organoid systems that will arise as the field advances. To this end, we propose a pipeline for establishing, characterizing and benchmarking future organoids, in turn promoting the standardization and validation of organoid models. Along the way, we provide a detailed overview of the recent progress in the culture of organoids derived from the liver, biliary tree and pancreas, as well as PSC-derived organoids differentiated towards the hepatic, biliary and pancreatic 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 primary cells, stem/progenitor cells or pluripotent stem 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). This contrasts the previous understanding 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, 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. In

the context of HBP organoid biology, these include epithelial organoids, multi-tissue organoids, and multi-organ organoids (Figure 1).

Epithelial organoids represent the most developed and intensely studied organoid system. Currently exclusive to this subtype, is the ability to self-renew under the appropriate culture conditions. When applied to organoids, self-renewal describes the *repeated regeneration of complete organoids from organoid fragments or single cells*, allowing for the expansion of cultures. 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 or enzymatic and/or chemical fragmentation of epithelial organoids into disordered cell clusters (followed by secondary culture in expanding conditions), cells reorganize and/or proliferate, generating complete organoids (Figure 2) (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 (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 synchronize with the proliferative state of the organoid.

Multi-tissue organoids (Figure 1) are established through the co-culture of endodermal and mesodermal stem/progenitor cells, or the co-differentiation of PSCs (Ouchi *et al.*, 2019; Takebe *et al.*, 2015; Wu *et al.*, 2019). 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 co-differentiate to attain a stable level of maturity and function (Ouchi *et al.*, 2019; Takebe *et al.*, 2017). An advantage of multi-tissue organoids is their native, tissue-like, hetero-cellular composition. Multi-tissue organoid systems are especially well placed to study the heterotypic cell-cell interactions of multiple cell types normally present in the native tissue. Importantly, these cultures show intra-organ self-organization between endodermal and mesodermal components, similar to that of the native tissue (Takebe *et al.*, 2013).

Multi-organ organoids (Figure 1) are the most complex and least described organoid type, with only one report in the context of HBP organoids (Koike *et al.*, 2019). Characteristic to this sub-type is inter-organ developmental self-organization patterns. As demonstrated by Koike *et al.*, these systems hold great promise in the study of organogenesis, a process governed by several boundary tissue interactions (Strand *et al.*, 2010). Notably, HBP multi-organ organoids could be maintained in culture for at least 90 days, not only displaying multiple HBP 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, but discourage cell-substrate/matrix interaction (Cui *et al.*, 2017). As a result, cells interact to form a compact sphere, although other shapes are possible. This is in contrast to most organoid systems, which form when placed in a matrix-rich 3D environment with which cells can interact (Huch and Koo, 2015; Tysoe *et al.*, 2019). That withstanding, while there is no predefined matrix with which cells can interact during the self-organization of spheroids, ECM cues *are* involved in their formation. These take the form of long chain ECM fibers containing multiple RGD domains present on cell surfaces (Cui *et al.*, 2017). Furthermore, cells secrete their own ECM molecules which likely participate in their organization. Notably, some multicellular spheroid systems have been shown to recapitulate architectural and functional aspects of the original tissue (Bell *et al.*, 2016). 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 (Figure 1).

Hepatic, Biliary and Pancreatic Organoids Derived from Pluripotent Stem Cells

Since Yamanaka and colleagues reported the efficient reprogramming of mature somatic cells into iPSCs in 2007 (Takahashi *et al.*, 2007), numerous protocols have been developed to direct the differentiation of iPSCs to specific cell types (Osakada *et al.*, 2009; Spence *et al.*, 2011; Wong *et al.*, 2012). While early endeavors focused on stepwise differentiation protocols exclusively in 2D (Kunisada *et al.*, 2012; Palakkan *et al.*, 2017), there has been a shift towards 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 HBP organoids from iPSCs. 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 following paragraphs we review the recent progress in the culture of hepatic, biliary and pancreatic 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.* reported the generation of multi-tissue “liver bud” organoids from the co-culture of iPSC-derived hepatic endodermal progenitors, HUVECs and MSCs (Takebe *et al.*, 2013). 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. *In vivo*, the 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.* established a blend of morphologically diverse iPSC-derived hepatic epithelial organoids (Guan *et al.*, 2017). Interestingly, organoids were predominantly composed of either (a) hepatocyte-like-cells (HLCs), (b) cholangiocyte-like-cells (CLCs), or (c) mixed, containing both HLCs and CLCs. Depending on the culture conditions, organoids could be expanded as self-renewing cystic structures, or matured to non-proliferating HLCs. Functional analysis revealed that organoids were competent to perform mature 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 (Guan *et al.*, 2017).

In a novel approach, Wu *et al.* generated hepatobiliary multi-tissue organoids from iPSCs by simultaneously inducing both endodermal and mesodermal differentiation. Notably, 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). More recently, Ouchi *et al.* developed a method to generate hepatic multi-tissue organoids from iPSCs or ESCs. Strikingly, single-cell 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.* (Wang *et al.*, 2019) and Akbari *et al.* (Akbari *et al.*, 2019) established novel protocols for the derivation and culture of self-renewing ESC- and iPSC-derived hepatic epithelial organoids, respectively. In both cases organoids expanded as epithelial cysts, morphologically resembling the bile duct-derived organoids described by Huch *et al.* (Huch *et al.*, 2015). Notably, the ESC-derived hepatic organoids described by Wang *et al.* were able to engraft and repopulate a significant proportion of the liver parenchyma when transplanted into $Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$ (FRG) mice. Furthermore, when the organoids were ectopically transplanted into immunocompromised mice, they remained restricted to the hepatic lineages and displayed no oncogenic potential (Wang *et al.*, 2019).

In a recent breakthrough, Bin Ramli *et al.* 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 reveled 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. Notably, treatment of organoids with the cholestasis-inducing drug, troglitazone, disrupted the bile canaliculi network (Bin Ramli *et al.*, 2020).

PSC-derived Biliary Organoids

In 2014 Dianat *et al.* described the differentiation of ESCs and iPSCs into functional CLCs. When cultured in 3D conditions, PSC-derived CLCs formed cysts with baso-lateral polarization and mature cholangiocyte functions. 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. These PSC-derived cholangiocyte organoids displayed key functions and were successfully used to model genetic diseases affecting the bile duct epithelium, such as cystic fibrosis and Alagille syndrome. Notably, researchers were able to rescue the disease phenotypes with pharmacological intervention, validating them as a drug screening tool. Furthermore, organoids were 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.* described the generation of pancreatic epithelial organoids from PSC-derived pancreatic exocrine progenitors. Morphologically, organoids took the form of 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, organoids could be matured *in vitro* by modifying the culture protocol, or *in vivo* following transplantation into immunodeficient mice. In both cases organoids formed pancreatic exocrine structures containing $CPA1^+$ acinar and $KRT19^+$ ductal compartments (Huang *et al.*, 2015).

Two years later Hohwieler *et al.* reported the differentiation of iPSCs and ESCs to pancreatic progenitors and their culture as epithelial organoids. Notably, 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 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 pancreatic organoids 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 engraft, with signs of neovascularization and tri-lineage differentiation, including insulin producing cells (Hohwieler *et al.*, 2017).

More recently, Yoshihara *et al.* 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 NOD/SCID mice, HLIOs quickly reestablished glucose hemostasis. Notably, through the overexpression of the immune checkpoint protein, programmed death-ligand1 (PDL1), HILOs were even able to engraft and 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) .

Organoids Derived from Primary Tissue of the Human Liver, Biliary Tree and Pancreas

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), extrahepatic biliary tree (Lugli *et al.*, 2016; Sampaziotis *et al.*, 2017) and pancreas (Broutier et al., 2016; Georgakopoulos et al., 2020; Loomans et al., 2018) (Figure 3). To establish cultures, isolated cells or tissue fragments are embedded in a matrix-rich 3D environment, typically Engelbreth-Holm-Swarm (EHS)-derived hydrogels, and supplemented with medium containing important growth factors and small molecules. Within days, 3D structures begin to arise that can be serially passaged for several months (Broutier *et al.*, 2016; Tysoe *et al.*, 2019). Notably, tissue-derived epithelial organoids display high levels of genetic stability and are committed to their tissue of origin, making them an extremely 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 hepatic, biliary and pancreatic organoids.

Building a Consensus on Nomenclature of Tissue Derived Epithelial Organoids

Consistency is currently lacking when referring to tissue-derived HBP organoids. For example, the intrahepatic bile duct-derived epithelial organoid systems are commonly referred to as *liver organoids* (Huch *et al.*, 2015), *ductal organoids* (Prior *et al.*, 2019a), and *cholangiocyte organoids* (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.

In building a consensus on the nomenclature of HBP 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 answering 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 the methods. In this way you know where you begin (cell and tissue of origin) — and where you end (cell type of resemblance *in vitro*). In some cases, the two align. For example, the intra- and extrahepatic cholangiocyte organoid systems 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 established by Hu *et al.* expand as condensed structures with typical hepatocyte morphology. However, when cultured under the conditions established by Huch *et al.* for the culture of intrahepatic cholangiocyte organoids, cells transdifferentiate and reorganize, expressing classical cholangiocyte markers and taking on a cystic morphology (Hu *et al.*, 2018). Recent discoveries have highlighted the extensive degree of plasticity that exists between the epithelial cells of the mouse liver, with numerous reports of transdifferentiation

between hepatocytes and cholangiocytes *in vivo* (Gadd *et al.*, 2020; Michalopoulos *et al.*, 2005; Raven *et al.*, 2017; Schaub *et al.*, 2018). However, whether this phenomenon also occurs in humans has yet to be established.

For epithelial organoid systems in which *multiple cell types* arise, it was decided that the nomenclature should reflect the anatomical structure that arises. Clear examples of these systems include the gut organoids that arise from single $Lgr5^+$ 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 $Procr^+$ 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. We anticipate the establishment of more transdifferentiating and multi-cell type epithelial organoids systems in the years to come.

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 all organs (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 reaction (Planas-Paz *et al.*, 2019). Interestingly, in 2013 Huch *et al.* described a protocol for the long-term, clonal expansion of single mouse $Lgr5^+$ liver ductal cells as organoids. It was proposed that the organoid initiating cells were derived from a rare $Lgr5^+$ stem cell population of ductular origin that became activated upon carbon tetrachloride (CCl_4) injury, which selectively damages pericentral hepatocytes (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 liver and pancreas progenitors, leaving the question open as to whether the cell of origin was indeed a cholangiocyte, or a *bona fide* 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 which 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.* demonstrated that Lgr5 mediated Wnt/β-catenin signaling is absent and dispensable for EpCAM$^+$ cholangiocytes to initiate a ductular reaction *in vivo*. However, upon *in vitro* culture under Wnt/B-catenin-inducing conditions, isolated EpCAM$^+$ cholangiocytes form organoids and upregulate Lgr5 (Planas-Paz *et al.*, 2019). Notably, mouse intrahepatic cholangiocytes cultured under the described conditions express multiple progenitor, hepatocyte and cholangiocyte markers, suggestive of a bipotential nature. Indeed, upon modifying the culture conditions to stimulate hepatic maturation, mouse intrahepatic cholangiocyte organoids (mICOs) expressed markers of mature hepatocytes and could perform some hepatocyte functions (Huch *et al.*, 2013a). Interestingly, Prior *et al.* recently demonstrated that Lgr5 marks a sub-population of truly bi-potent hepatoblasts in the early mouse embryo (E9.5) (Prior *et al.*,

2019). Whether the ability of adult cholangiocytes to initiate organoids is restricted to a select subset of cells with increased plasticity, or equally shared amongst biliary epithelial cells (BECs) remains to be determined.

In 2015 the culture of mICOs was adapted to support the culture of human intrahepatic cholangiocyte organoids (hICOs). To do this, the medium composition was adapted to include the cAMP pathway agonist, forskolin (FSK) and inhibitor of TGFβ receptors ALK4/5/7, A8301. Under these conditions hICOs were highly proliferative, expanding for several months while remaining genetically stable. Notably, hICOs expressed a mixture of stem cell markers (LGR5 and SOX9), hepatocyte markers (HNF4α) and ductal markers (KRT19 and Onecut2). However, under expansion conditions organoids displayed a biliary phenotype and failed to express markers of mature hepatocytes, such as albumin or CYP3A4. In order to exploit the suspected bipotential character of the cells and achieve hepatocyte differentiation, media was developed that lacked R-spondin and FSK and included BMP7, FGF19, dexamethasone and the Notch inhibitor, DAPT. In 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, drug metabolism and ammonia detoxification. Notably, 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).

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.* demonstrated that morphologically indistinguishable organoids could be established from the fragments of gallbladder and extrahepatic bile duct tissue, although they did not provide a detailed characterization of the human gallbladder cholangiocyte organoids (hGCOs). Mouse GCOs expressed stem cell markers Prom1, Sox9 and Lgr5, as well as biliary makers Cldn3, EpCAM and Itga6. To confirm the origin of the organoids, expression of 413 gallbladder-specific and 190 liver-specific genes was analyzed. Importantly, mGCOs expressed the gallbladder, but not the liver specific genes and had gene expression profiles that distinguished them from mICOs (Lugli *et al.*, 2016).

Extrahepatic Cholangiocyte Organoids (ECOs)

In 2017 Sampaziotis *et al.* developed a novel protocol to culture human cholangiocytes derived from the extrahepatic biliary tree 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 (ERCP), a minimally invasive procedure. To initiate ECOs, isolated cholangiocytes were embedded in Matrigel and cultured in medium supplemented with 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 into 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-scaffolds were transplanted into mouse models of CBD and gallbladder injury. Notably, mice transplanted with the resulting tissue constructs were capable of surviving 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 CFTR (Sampaziotis *et al.*, 2017).

Regional Diversity of Cholangiocyte Organoids

In an attempt to better understand the differences between cholangiocyte organoids (COs) derived from different regions of the biliary tree, Rimland *et al.* established ICOs, ECO 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 direct comparison. Interestingly, gene and protein expression analyses revealed that ICO, ECOs and GCOs are remarkably similar, despite some regional differences corresponding to the anatomical location from which cells are isolated. Of note, only ICOs demonstrated the ability to upregulate hepatocyte markers upon *in vitro* differentiation (Rimland *et al.*, 2020). From an embryological perspective, the selective capacity of ICOs to upregulate hepatocyte markers makes sense. Intrahepatic cholangiocytes arise from bipotential hepatoblasts, while extrahepatic cholangiocytes arise from the caudal portion of the hepatic diverticulum (Spence *et al.*, 2009). This could imply that ICOs, but not ECOs have the epigenetic landscape to support hepatocyte (trans)differentiation.

Hepatocyte Organoids (HOs)

In 2018 Peng *et al.* reported the long-term 3D culture of primary mouse hepatocytes as organoids. To establish mouse hepatocyte organoids (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, TNFα. While the study only describes the culture of mouse hepatocyte organoids (mHOs), 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, TNFα activates a series of transcription factors including NF-κB, JAK/Stat, AP-1 and YAP, which enhance cell proliferation (Peng *et al.*, 2018).

Under the expansion conditions described, mHOs could be expanded for at least 8 months. Withdraw of TNFα 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 (Figure 4). Periportal (PP) hepatocytes (zone 1) are specialized in β-oxidation and gluconeogenesis, whereas pericentral (PC) hepatocytes (zone 3) are better capable of 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 mediums: one *with* Wnt/β-catenin activation and one *without* Wnt/β-catenin activation, to separately induce the expression of either PP or PC genes, allowing for a zonally defined maturation of mHOs. Functionally, mHOs in both DMs secreted albumin, with the highest levels in the PP-induction medium. Moreover, in both DMs mHOs were functional in LDL uptake, canicular transport and glycogen storage. Upon transplantation into $FAH^{-/-}$ 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.* 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. IF staining showed that mHOs had strong Alb expression and were negative for the cholangiocyte markers KRT19 and KRT7. 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 showed that single embryonic mouse $Lgr5^{+}$ bi-potent hepatoblasts could be expanded *in vitro* and fated either to the cholangiocyte or hepatocyte lineage, depending on the culture conditions. When cultured in hICO expansion medium, hepatoblasts would form cholangiocyte organoids. However, when cultured in the hepatocyte medium for expanding human hepatocytes in 2D (Zhang et al., 2018), cells would form hepatocyte organoids (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. Fetal liver-derived organoids were distinct from cholangiocyte organoids (COs) and morphologically resembled mHOs. Transmission EM 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 ending up in small lumens was observed within organoids, indicating that cells were not only polarized, but also interconnected. Similar to mHOs, transcriptomic and functional analysis of fetal-liver derived organoids was more comparable to primary human hepatocytes than to cholangiocytes (Hu *et al.*, 2018). It should, however, be noted that the cellular origin of human fetal liver-derived organoids was not demonstrated. As the plating efficiency was low (<1%), it is possible a rare stem/progenitor cell is the cell-of-origin of fetal liver-derived organoids.

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 major 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.

Pancreatic Organoids (POs)

In 2013 the group of Anne Grapin-Botton demonstrated that embryonic mouse pancreatic progenitors can be cultured as self-organizing 3D structures with tri-lineage (acinar, ductal and endocrine) differentiation potential, although these cultures could not be propagated long term (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 which lacked an endocrine compartment. However, following transplantation endocrine differentiation was stimulated (Huch *et al.*, 2013b).

In 2015, the culture of mouse pancreatic ductal organoids (mPDOs) was adapted to support human pancreatic ductal adenocarcinoma organoids (PDACOs), as well as non-neoplastic pancreatic ductal organoids (hPDOs)(Boj *et al.*, 2015). Since their establishment, culture conditions for hPDOs have improved. With the removal of Wnt ligands, and the addition of PGE_2 and FSK, hPDOs could be mass expanded long-term (Georgakopoulos *et al.*, 2020). Notably, under these conditions hPDOs resembled the primary tissue closely on a gene expression level, with the exception that Wnt-target and progenitor markers were significantly upregulated *in vitro*. When initiated from single pancreatic ductal cells, hPDOs resembled cystic spheroids, with occasional budding structures along the periphery of a central lumen composed of a polarized monolayer of epithelial cells (Georgakopoulos *et al.*, 2020). Interestingly, acinar cells (the other cell type of exocrine system) have also been cultured as organoids under similar conditions. However, upon *in vitro* expansion of single acinar cells, cells gradually lost their acinar-cell expression and transdifferentiated into pancreatic ductal organoids (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, $ALDH^{hi}$ expressing cells localized in the tips of the budding structures showed progenitor characteristics and could be partially differentiated towards 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 protein C receptor (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, FG2, heparin and VEGFa. When analyzed by single-cell RNA sequencing (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).

We anticipate the establishment of novel and more complex pancreatic organoids and we propose nomenclature and standardization for these systems in the supplementary data (Table

S3).

Organoids Derived from Primary and Metastatic Tumors of the Liver, Biliary Tree and Pancreas

Definition and Nomenclature for Tumor Derived Organoids

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), biliary tree (Saito *et al.*, 2019) and pancreas (Boj *et al.*, 2015) could also be cultured as self-renewing 3D structures. Similar to non-tumor epithelial organoids, tumor-derived organoids self-organize through cell-cell and cell-matrix interactions. However, rather than recapitulating aspects of the healthy tissue, tumor-derived organoids capture the histological organization of the native tumor. Notably, tumor organoids 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 tumor-derived organoids 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 and Table S4). In this section we review the recent progress in the culture of tumor organoids derived from the liver, biliary tree and pancreas.

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), as demonstrated by the group of Huch *et al.* (Broutier *et al.*, 2017). Notably, malignant cells possessed tumor-driving mutations, which allowed for their selective outgrowth compared to non-transformed cells. Morphologically, HCCOs resembled dense spheroidal structures. Typical HCC markers, such 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 demonstrated that upon subcutaneous transplantation of HCCOs, novel tumors were formed, demonstrating the HCCOs retain their oncogenic potential after *ex vivo* culture (Broutier *et al.*, 2017). A subsequent study by Nuciforo *et al.* demonstrated that similar organoids could be cultured from needle-biopsies of the tumor, making HCCOs available to a broader patient population. Since only a minority of patients would receive a surgical resection and patients receiving resection are less likely to receive systemic therapies (Forner *et al.*, 2018), tumor-needle biopsies represent a convenient tissue source.

Tumor Organoids Derived from Cholangiocarcinoma

In the same publication in which Broutier *et al.* described the culture of HCCOs, the feasibility of

culturing neoplastic epithelial cells from intrahepatic cholangiocarcinomas (iCCAs) as organoids was also demonstrated. Intrahepatic cholangiocarcinoma 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, while HCC markers such as albumin and AFP were absent. Similar to HCCOs, iCCA organoids retained the mutational landscape of the original tumor. Notably, when iCCAOs were subcutaneously transplanted in mice, they formed tumors with 100% efficiency (37/37 attempts, n=2 CCA lines), taking on glandular structures similar to primary iCCAs (Broutier *et al.*, 2017).

Building on this work, Saito *et al.* established tumor organoids from other regions of the biliary tree and pancreas. These included gallbladder carcinoma organoids (GBCOs), pancreatic ductal adenocarcinoma organoids (PDACOs), and a 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) the authors noticed that non-tumor derived organoids proliferated faster than tumor organoids (Saito *et al.*, 2019). Thus, novel medium formulations that promoted selective outgrow of tumor cells were devised. 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 Clevers lab demonstrated the feasibility of culturing tumorous lesions, both primary and metastatic, as tumor organoids (Boj *et al.*, 2015). Unfortunately, most pancreatic ductal adenocarcinomas (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 pancreatic ductal adenocarcinoma organoids (PDACOs), 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 feasibility to use these organoids to model disease progression.

More recently Seino *et al.* 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. During subtype analysis, the group could identify a (1) non-Wnt-producing subtype, which was demonstrated to require Wnt activation provided by associated fibroblasts, (2) a Wnt-secreting subtype and (3) R-spondin and Wnt-independent subtype. Interestingly, it was demonstrated that Wnt-producing subtypes were associated with increased disease progression, metastatic potential and that Wnt-niche independency was regulated by GATA6 (Seino *et al.*, 2018).

Standardization and Validation of HBP Organoids (*The Pipeline*)

Numerous reports describe contrasting methods of generating HBP epithelial organoids from the same cell source (Broutier *et al.*, 2016; Lugli *et al.*, 2016; Rimland *et al.*; Tysoe *et al.*, 2019) (Table S2). 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. Nikolaev *et al.* 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 (Nikolaev *et al.*, 2020). 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 a pipeline (Figure 6) for researchers to follow when establishing a novel system or refining an existing one.

First, researchers should strive to define 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 (FACS), 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 both a gene expression and functional level, both before and after long-term culture. When possible, we encourage the use of emerging technologies, such as single-cell analysis. When conducting these analyses, the organoid system should be benchmarked against the tissue and cell of origin/interest and compared to previously established systems. To this end, the construction of an open source, high quality data set containing single-cell data of HBP primary tissue and the corresponding organoids would be of great value. Finally, we encourage the use of recombinant growth factors or small molecules, rather than conditioned medium in the culture of organoids when possible. We believe the described pipeline will promote the standardization and validation of organoid systems, optimized to answer the research question at hand.

Clinical Application of HBP 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 at all steps. 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 reliability and reproducibility of results more difficult. Thus, it is imperative to find a replacement for EHS-based materials in order to move organoids towards the clinic, as well as promote the standardization and validation of organoid models.

Notably, several efforts have been made to this end. For instance, Broguiere *et al.* 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.* utilized synthetic polyisocyanopeptides (PIC) 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.* reported not only the culture, but also the derivation of ICOs in PEG 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.*, 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 5 passages) of human pancreas organoids in the optimized human ductal pancreas medium (Georgakopoulos *et al.*, 2020). In another approach, Giobbe *et al.* demonstrated that ECM-hydrogels derived from decellularized tissues could support the formation and growth of endoderm-derived human organoids (Giobbe *et al.*, 2019).

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.* utilized commercial spinner flasks to mass expand intrahepatic cholangiocyte organoids (ICOs). Notably, ICOs expanded in stirred suspension achieved an average of a 43-fold induction in two weeks, compared to a 6-fold increase in cell number in static cultures (Schneeberger *et al.*, 2020). In another study, Kumar *et al.* used orbital shakers (60 rpm) 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). While 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

Finally, for organoids to make their way to the clinic they must be immunocompatible. While 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 autologously isolated by minimally invasive methods and mass expanded *ex* vivo (Sampaziotis *et al.*, 2017). Recently, Soroka *et al.* demonstrated that COs could be established from bile, circumventing the need for patient biopsies (Soroka *et al.*, 2019). However, minimally invasive isolation methods are not available for each type of organoid. Furthermore, 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.*, demonstrated that pulses of interferon-gamma induced the expression of PD-L1 in islet organoids, allowing them to avoid immune destruction when transplanted into diabetic mice with a functional immune system. PD-L1 expressing organoids provided sustained blood sugar control without the need for genetic manipulation for at least 40 days (Yoshihara *et al.*, 2020).

Concluding Remarks

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

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Figures

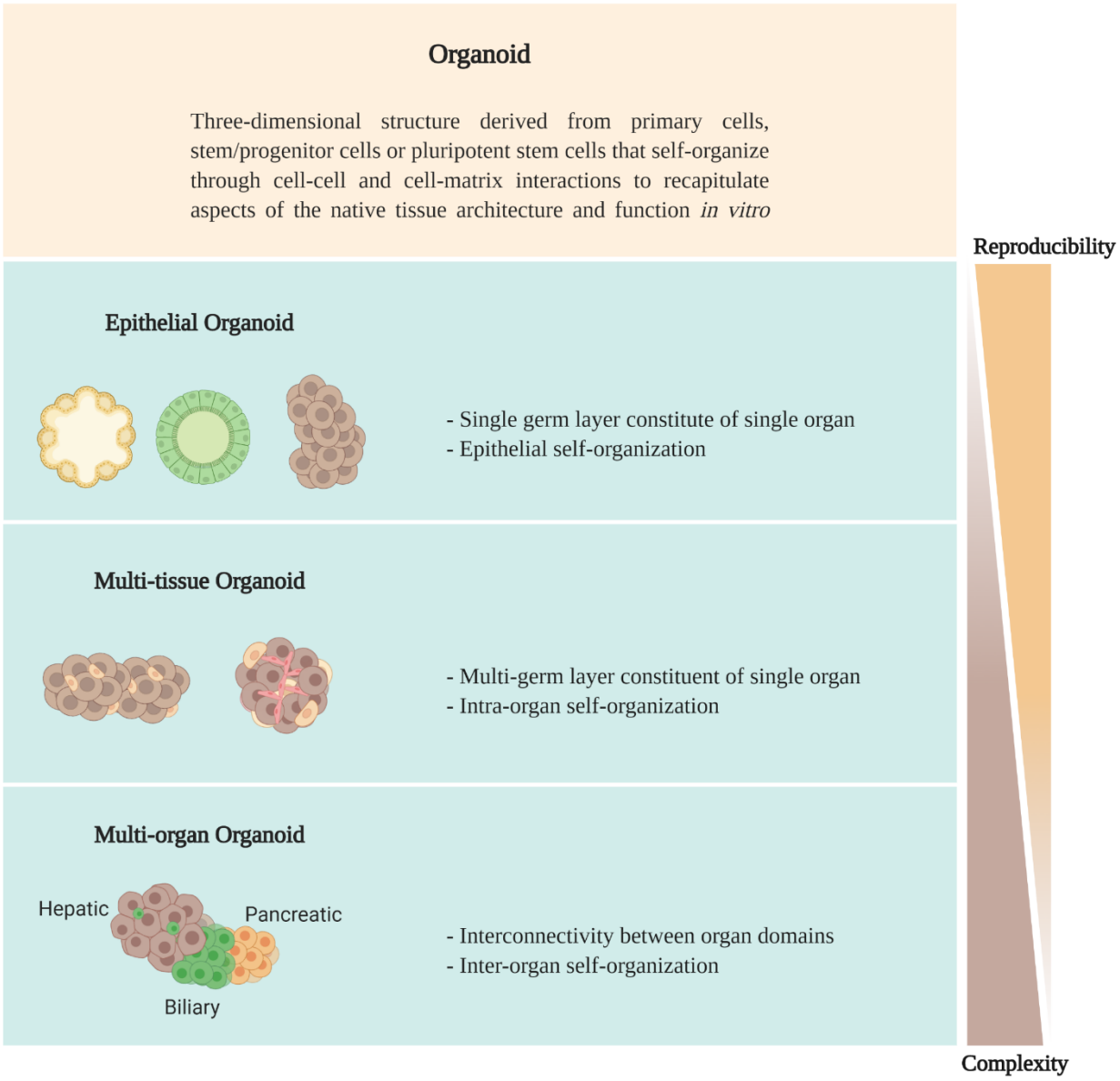

Figure 1. Overarching definition of an organoid (top panel) along with the three sub classifications in the contexts of HBP organoids

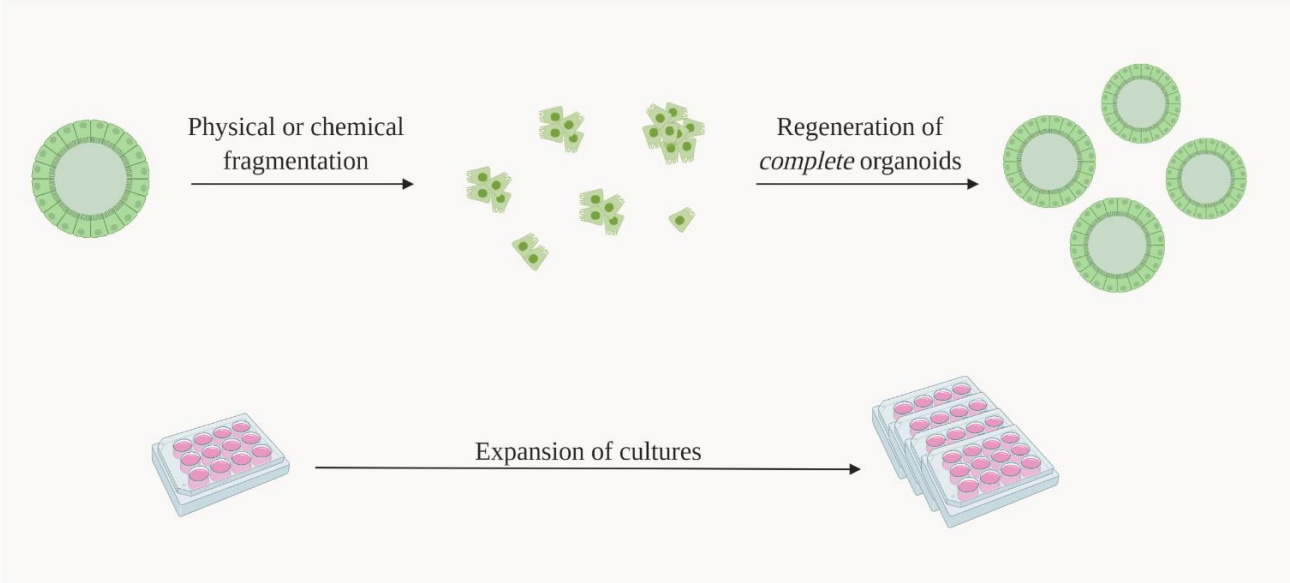

Figure 2. Self-renewal of epithelial organoids. Upon physical or chemical disruption of organoids into fragments or single cells, followed by secondary culture in expanding conditions, cells reorganize and expand, generating complete organoids.

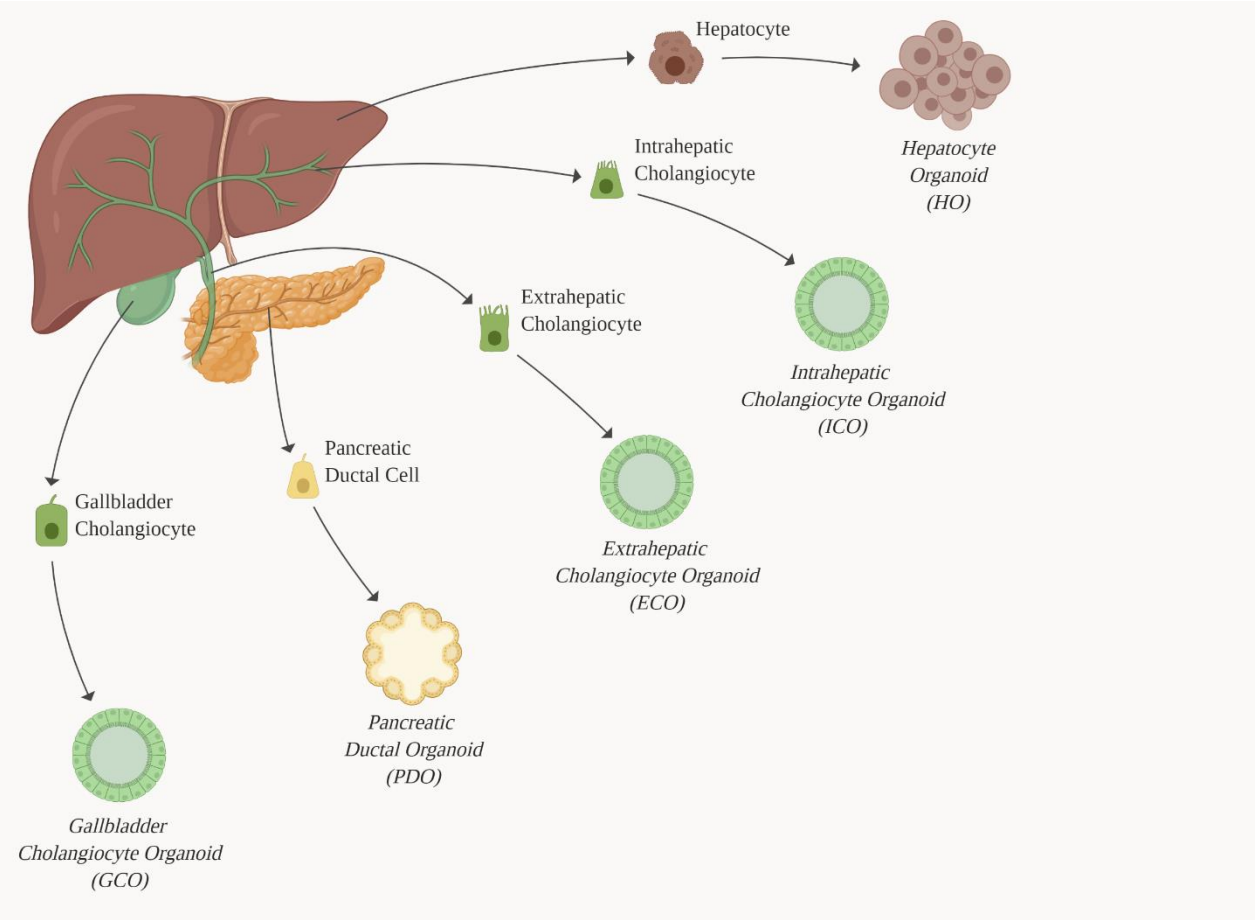

Figure 3. Nomenclature for epithelial organoids derived from primary tissue of the liver, biliary tree and pancreas

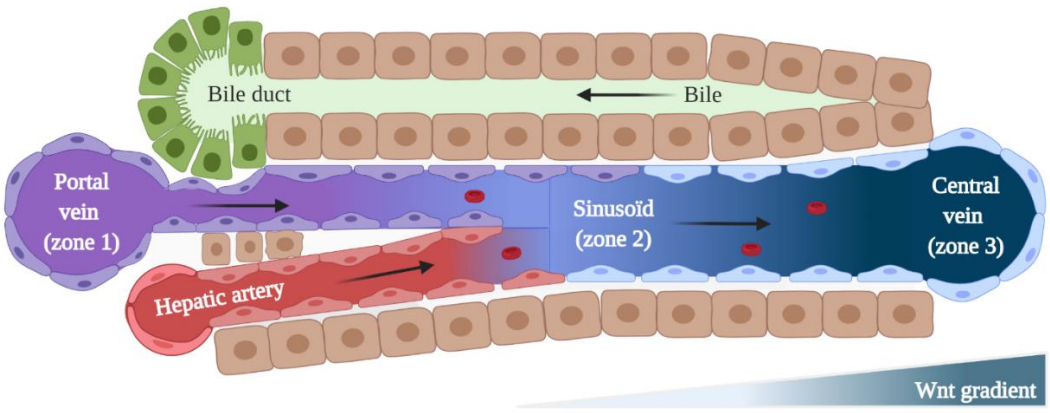

Figure 4. Schematic overview of hepatocyte zonation in the liver, driven largely as a result of pericentral vein derived Wnt gradient

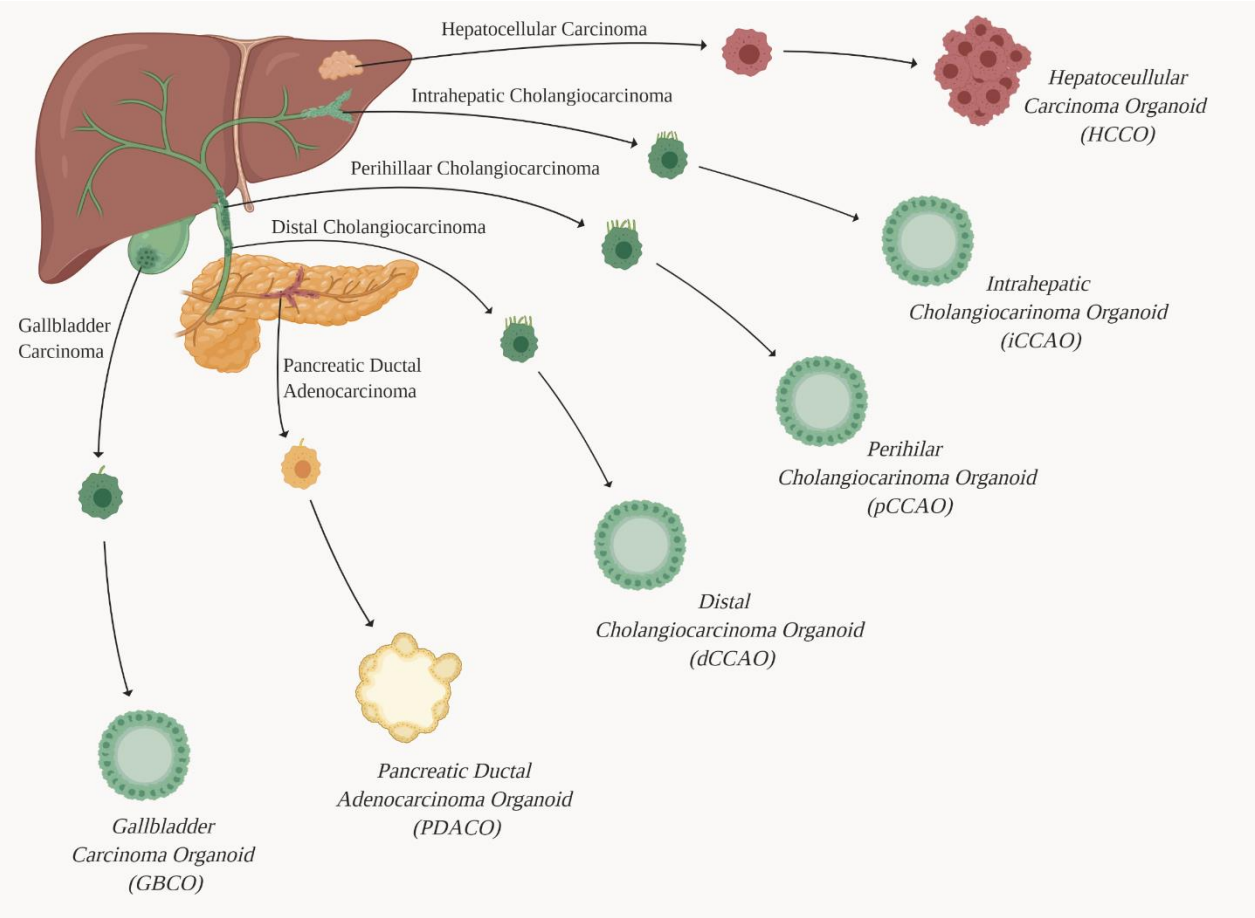

Figure 5. Nomenclature for tumor organoids derived from primary or metastatic tumors of the liver, biliary tree and pancreas

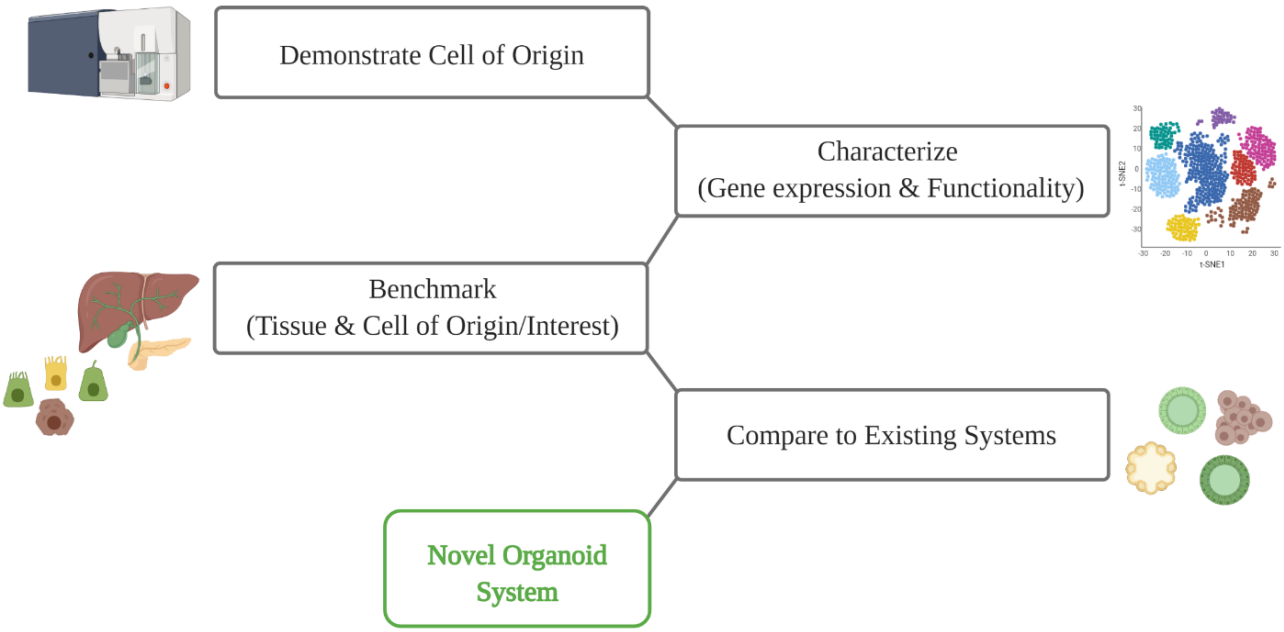

Figure 6. Pipeline for the establishment of novel organoid system.

Supplementary Information

Supplementary Methods

Expert Invitation

Scientists (hereafter referred to as experts) were invited based on a PUBMED and Google Scholar database search for HBP organoids, regeneration and development. Experts were invited if they had made a significant contribution (either 1st, 2nd or senior authorship) to a peer-reviewed, published manuscript retrieved from the search. From the generated list of experts, select authors with extensive expertise in HBP organoids, regeneration or development were invited to join the steering committee (for members of the steering committee see supplementary table 1). Once invited, all experts had the option to suggest additional experts which would be evaluated by the core team initiating this effort (AM, FJMR, MMAV, LvdL and BS) before receiving an invitation. In a similar fashion, these experts had the option to suggest experts as well. After the first the questionnaire round, no additional experts were eligible for invitation.

Building Consensus

To reach consensus, a modified Delphi method based on three successive questionnaires was employed. Consensus was defined as ≥90% agreement on a single question. Questions for which consensus was reached were removed from subsequent questionnaires. Additionally, if an option to a question received <15% of the votes from the expert panel, it was removed from the subsequent questionnaires. Experts had the option to suggest additional answers for each question during the first and second questionnaire. If a suggestion was mentioned by three or more experts, this suggestion was added as an option in the following questionnaire. Furthermore, experts had the option to make additional remarks in the questionnaire, as well as have an open discussion with the core team on their views regarding consensus statements. After each questionnaire, a summary document of the answers was created and sent out to the participating experts, along with an invitation to complete a new questionnaire. Importantly, as per the Delphi method the results of each questionnaire were anonymous. Topics for which consensus was not reached after the third and final questionnaire were deliberated by the steering committee through a round table discussion. Based on the discussion, a final proposition was made by the steering committee for these questions. All experts were given time to review the propositions made by the steering committee, along with the opportunity to discuss their views.

Questionnaires

Questionnaires were created in google documents (Alphabet Inc., CA). The initial draft of each questionnaire was designed by the core team and evaluated by the steering committee and dr. Sheila Chari before being sent out to invited experts. Each questionnaire was divided into four categories: (1) definition of an organoid, (2) nomenclature for tissue-derived organoids, (3)

organoids derived from fetal biopsies and (4) nomenclature for tumor-derived organoids. Each questionnaire was designed to reflect the results of the previous questionnaire. Questionnaires can be found in supplementary data file 1-3.

Supplementary Results

Questionnaire Responses

Based on the database search 61 authors were identified and subsequently invited to fill in the first questionnaire. An additional 39 authors were suggested by experts, making a total of 100 invitees. The first questionnaire was filled in by 74 experts (response of 74%). Experts which completed the first questionnaire were invited to fill in the second questionnaire. Out of the 74 eligible candidates, 69 (93%) completed the second questionnaire. Similar to the previous round, experts which filled in the second questionnaire were invited to complete the third and final questionnaire. In the end, 68 experts (see supplementary table 1) representing 16 countries around the world (Figure S1) participated in all three questionnaires.

Supplementary Figures and Tables

Figure S1. Countries represented in the HBP Consortium.

Table S1. Members of the HBP Consortium

	Name	Affiliation	Country	Role
1	Ary Marsee	Utrecht University	The Netherlands	Core team
2	Floris Roos	Erasmus MC	The Netherlands	Core team
3	Monique Verstegen	Erasmus MC	The Netherlands	Core team
4	Hans Clevers	Hubrecht Institute	The Netherlands	Steering committee
5	Ludovic Vallier	University of Cambridge	United Kingdom	Steering committee
7	Takanori Takebe	Cincinnati Children's Hospital and Tokyo Medical Dental University	USA and Japan	Steering committee
6	Meritxell Huch	Max Planck Institute of Molecular Cell Biology and Genetics	Germany	Steering committee
8	Weng Chuan Peng	Princes Maxima Center	The Netherlands	Steering committee
9	Stuart Forbes	MRC Centre for Regenerative Medicine	United Kingdom	Steering committee
10	Frédéric Lemaigre	Institut de Duve	Belgium	Steering committee
11	Eelco de Koning	Hubrecht Institute	The Netherlands	Steering committee
12	Helmuth Gehart	Institute for Molecular Health Sciences	Switzerland	Steering committee
13	Luc van der Laan	Erasmus MC	The Netherlands	Core team
14	Bart Spee	Utrecht University	The Netherlands	Core team
15	Sylvia Boj	Hubrecht Organoid Technology (HUB)	The Netherlands	HBP Consortium
16	Pedro Baptista	Aragon Health Sciences Institute	Spain	HBP Consortium
17	Kerstin Schneeberger	Utrecht University	The Netherlands	HBP Consortium
18	Carol Soroka	Yale University	United States	HBP Consortium
19	Markus Heim	University of Basel	Switzerland	HBP Consortium
20	Sandro Nuciforo	University of Basel	Switzerland	HBP Consortium
21	Kenneth Zaret	University of Pennsylvania	United States	HBP Consortium
22	Yoshimasa Saito	Keio University School of Medicine	Japan	HBP Consortium
23	Matthias Lutolf	LSCB – Laboratory of Stem Cell Bioengineering - EPFL	Switzerland	HBP Consortium
24	Vincenzo Cardinale	University of Roma	Italy	HBP Consortium
25	Ben Simons	University of Cambridge	United Kingdom	HBP Consortium

26	Sven IJzerdoorn	University of Groningen	The Netherlands	HBP Consortium
27	Akihide Kamiya	Tokai University	Japan	HBP Consortium
28	Hiromi Chikada	Tokai University	Japan	HBP Consortium
29	Shuyong Wang	Beijing Institute of Health Service and Transfusion Medicine	China	HBP Consortium
30	Seon Ju Mun	Korea Research Institute of Bioscience and Biotechnology	South Korea	HBP Consortium
31	Myung Jin Son	Korea Research Institute of Bioscience and Biotechnology	South Korea	HBP Consortium
32	Tamer Tevfik Onder	Koc University	Turkey	HBP Consortium
33	James Boyer	Yale University	United States	HBP Consortium
34	Toshiro Sato	Keio University School of Medicine	Japan	HBP Consortium
35	Nikitas Georgakopoulos	University of Cambridge	United Kingdom	HBP Consortium
36	Andre Meneses	Universidade Federal Rural da Amazônia	Brazil	HBP Consortium
37	Laura Broutier	Cancer Research Centre of Lyon	France	HBP Consortium
38	Luke Boulter	University of Edinburgh	United Kingdom	HBP Consortium
39	Dominic Grün	Max Planck Institute of Immunology and Epigenetics	Germany	HBP Consortium
40	Jan IJzermans	Erasmus MC	The Netherlands	HBP Consortium
41	Benedetta Artegiani	Prinses Maxima Centrum	The Netherlands	HBP Consortium
42	Ruben van Boxtel	Prinses Maxima Centrum	The Netherlands	HBP Consortium
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Table S2. Types of HBP Organoids

Organoid Type	Reference	Nomenclature	Abbreviation	Key characteristics
Cholangiocyte	(Huch et al., 2015; Lugli et al., 2016; Sampaziotis et al., 2017)	Cholangiocyte organoids. Depending on the region either intrahepatic (I), extrahepatic (E) or gallbladder (G)	ICOs, ECOs and GCOs	Markers: EpCAM$^+$, KRT7$^+$ KRT19$^+$, CFTR$^+$, GGT$^+$. Functional: ALP, GGT, CFTR and CaCC.
Hepatocyte	(Hu et al., 2018; Peng et al., 2018)	Hepatocyte organoids	HOs	Markers: Alb$^+$, ASGR1$^+$, HNF4α$^+$ Functional: Albumin synthesis, lipid metabolism and CYP functionality (drug metabolism and inducibility).
Liver	(Bin Ramli et al., 2020)	Liver organoids	LivOs	Consisting of both the hepatocyte and cholangiocyte compartments as described above, while showing functional integration and communication between both cell types. For instance, bile synthesis by hepatocytes which is transported towards the bile ducts.
Pancreatic ductal	(Boj et al., 2015; Georgakopoulos et al., 2020)	Pancreatic ductal organoids	PDOs	Markers: EpCAM$^+$, CFTR$^+$, SCTR$^+$. Functional: CFTR, SCTR, CaCC.
Pancreatic islet	(Wang et al., 2020)	Pancreatic islet organoids	PIOs	Consisting of either one or multiple cell types from a pancreatic islet as demonstrated by scRNA-seq. Functionality of individual cell types should be confirmed, for instance insulin synthesis by β-cells.
Hepatoblast	(Prior et al., 2019b)	Hepatoblast organoids	HBOs	Derived from fetal liver. Markers: Alb$^+$, AFP$^+$, TBX3$^+$. Functional: Bipotential differentiation (cholangiocytes and hepatocytes), while having limited lipid or drug metabolism when undifferentiated.

Table S3. Potential Future Pancreatic Organoids Systems

Organoid Type	Reference	Nomenclature	Abbreviation	Key characteristics
Endocrine pancreas	-	Endocrine pancreas organoids	EnPOs	Consisting of pancreatic islet along with cells from a different germ layer which functional integrate with one another.
Endocrine pancreas	-	Endocrine pancreas organoids	EnPOs	Consisting of pancreatic islet along with cells from a different germ layer which functional integrate with one another.
Pancreas	-	Pancreas organoids	POs	Combination of cells from both the exocrine and endocrine pancreas as described above. Both compartments should be present in the organoid and show functionality of both endocrine and exocrine pancreas.

Table S4. Definition and nomenclature of tumor-derived organoids

Tumor Type	Reference	Nomenclature	Abbreviation	Key characteristics
Cholangiocarcinoma (CCA)	(Broutier et al., 2017; Saito et al., 2019)	Cholangiocarcinoma organoids. Depending on the region either intrahepatic (I), perihilar (ph) or distal (d) should be mentioned.	iCCAOs, phCCAOs, dCCAOs	Derived from cholangiocarcinoma specimens. Should have similar tumor-mutations, protein-expression and functionality as the tumor sample it was derived from and be capable of tumor formation *in vivo.*
Hepatocellular carcinoma (HCC)	(Broutier et al., 2017; Nuciforo et al., 2018)	Hepatocellular carcinoma organoids	HCCOs	Derived from hepatocellular carcinoma specimens. Should have similar tumor-mutations, protein-expression and functionality as the tumor sample it was derived from and be capable of tumor formation *in vivo.*
Gallbladder carcinoma (GBC)	(Saito et al., 2019)	Gall bladder carcinoma organoids	GBCOs	Derived from gallbladder carcinoma specimens. Should have similar tumor-mutations, protein-expression and functionality as the tumor sample it was derived from and be capable of tumor formation *in vivo.*
Pancreatic ductal adenocarcinoma (PDAC)	(Boj et al., 2015)	Pancreatic ductal adenocarcinoma organoids	PDACOs	Derived from pancreatic ductal adenocarcinoma specimens. Should have similar tumor-mutations, protein-expression and functionality as the tumor sample it was derived from and be capable of tumor formation *in vivo.*