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Organoids and Regenerative Hepatology

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Abstract:

The burden of liver diseases is increasing worldwide, with liver transplantation remaining the only treatment option for end-stage liver disease. Regenerative medicine holds great potential as a therapeutic alternative, aiming to repair or replace damaged liver tissue with healthy functional cells. The properties of the cells used are critical for the efficacy of this approach. The advent of liver organoids has not only offered new insights into human physiology and pathophysiology, but also provided an optimal source of cells for regenerative medicine and translational applications. Here, we discuss various historical aspects of 3D organoid culture, how it has been applied to the hepatobiliary system, and how organoid technology intersects with the emerging global field of liver regenerative medicine. We outline the hepatocyte, cholangiocyte, and non-parenchymal organoids systems available and discuss their advantages and limitations for regenerative medicine as well as future directions.

Introduction:

The landscape of modern hepatology is rapidly shifting beneath our feet. Decades of steady progress in the areas of liver regeneration, stem cell biology, and cell culture techniques are now being exponentially accelerated by rapid advances in regenerative medicine, induced pluripotent stem cell (iPSC) technology, and organoid culture. These advances coincide with other explosive trends including large-scale genetic and epigenetic sequencing, bioinformatics, artificial intelligence, gene editing technology, and bioengineering. The confluence of these forces is quickly bringing previously unimaginable therapeutic options into focus and within reach as a new global field of regenerative hepatology is emerging. Regenerative hepatology is being developed as an alternative to liver transplantation for end stage liver disease. Indeed, despite recent advances in the management of liver disorders, transplantation remains the only curative treatment for advanced disease (1, 2). However, transplantation is limited by organ availability, complications, and the need for long term immunosuppression (1, 2). The goal of regenerative hepatology is to overcome these challenges, through two complimentary approaches. First, it aims to promote endogenous tissue repair and regeneration to reverse liver disease prior to irreversible damage. The second approach addresses cases where the liver's endogenous regenerative capacity has been exhausted, or the damage sustained is non-reversible (e.g., end-stage cirrhosis). In these cases, regenerative hepatology aims to replace the damaged cells or tissues with healthy ones in an

autologous or allogeneic manner. These approaches may be advantageous over liver transplantation for a variety of reasons. They are not limited by organ availability; autologous cells may be used, removing the need for immunosuppression; and cells can be administered as an infusion without need of an operation, limiting the risk of complications.

The liver is the most naturally regenerative organ in the human body and its remarkable regenerative properties have been recognized and studied for millennia, as referenced in the 7th century Greek myth of Prometheus. Because of the liver's astounding ability to tolerate long periods of injury and to regenerate after major parenchymal loss, the liver has always been a paradigm for human organ regeneration. Human liver can tolerate 70% hepatectomy while rodents can tolerate 90% hepatectomy. Regeneration of zebrafish liver can even occur after near complete obliteration of hepatocytes (3, 4). Understanding the mechanisms of liver regeneration is critical to the modern studies of cell and organ regeneration, longevity, and regenerative medicine. Indeed, several decades of intense research on liver regeneration have provided significant insights that can now inform the development of modern liver regenerative medicine approaches (5). It is also notable that, when the liver suffers severe or chronic damage, hepatocyte proliferation and liver regeneration are often attenuated. This failure of regeneration and lack of effective treatments for end-stage liver disease necessitates aggressive development of new regenerative strategies for rejuvenating injured tissue, stimulating, and supporting endogenous repair mechanisms, or replacing liver tissue with cell-based therapies or bioengineered tissue.

The strategies of regenerative medicine, for the liver as well as for other organs, can be captured through the R^3 paradigm which divides the aims of regenerative medicine into the repair triad of replacement, regeneration, and rejuvenation (6). Replacement involves transplantation of a cell-based therapy that re-establishes homeostasis (e.g., liver organoids, bio-engineered tissues) (7, 8). Indeed, the only clinically available regenerative medicine therapy for end-stage liver disease is, in fact, a replacement strategy (liver transplantation). In contrast, regeneration involves delivery and engraftment of stem cells or progenitor cells that then undergo growth and differentiation *in vivo* (e.g., stem cell transplant, stem-cell coated stents) (9-11). Lastly, rejuvenation involves inducing tissue self-renewal through activation of endogenous stem cells (e.g., gene therapy, exosome delivery) (12-14). Ongoing basic and translational research efforts, organized around these complementary strategies, are urgently needed to move forward. In addition to liver transplantation, platforms being developed include regenerative

pharmacotherapy, organoids as a cellular source of regeneration, and bioengineered liver tissue (Figure 2A).

Cellular therapies for end-stage liver disease have been explored for several decades, using primary (15, 16) hepatocytes and mesenchymal stromal cells (MSC) (17-19). These seminal studies set the foundation for the recent advances in regenerative hepatology with encouraging results. Primary hepatocyte transplantation was shown to be one of the first effective cellular therapies for acute liver failure (20). MSC injections have also been shown to improve inflammation and prevent disease progression (19). However, both approaches have limitations. Primary hepatocytes are hampered by limited availability and engraftment efficiency (21) but also manufacturing challenges, as these cells generally fail to proliferate and maintain their essential functions *in vitro* (16). MSCs show promising anti-inflammatory properties (22, 23) but the cells typically fail to engraft and replace damaged tissue (24, 25). Recent developments in liver organoid technology have been a turning point for addressing these challenges. In this review, we will focus on advances in regenerative hepatology using cell-based therapies and organoids while also including historical context, methodologies, nomenclature, applications, and clinical use cases.

Historical Aspects:

While organoid technology has undergone rapid adoption and significant advances in recent years, studies aimed at observing developmental biology and self-organization of cells *in vitro* actually began to emerge in the early to mid-1900's (26-28). Subsequently, various 3-dimensional (3D) cell culture systems were developed and have been in active use for many decades, including the hanging drop method (29), embryoid body formation (29), and cultures embedded in, or grown upon, extracellular matrix (ECM) (30). Late in the 20th century, scientists began to better simulate the physiological *in vivo* microenvironment by improving cell culture conditions. For example, breast epithelia when grown as 3D ducts were able to synthesize and secrete milk, as opposed to 2D culture conditions (31). Alveolar epithelial cells also differentiated more effectively in the presence of ECM (32). These studies highlighted the importance of cell-matrix interactions for tissue differentiation and maintenance. In 2008 Eiraku and colleagues generated polarized and functional cerebral cortex tissues from embryonic stem cells (ESCs) using 3D aggregate culture (33). Then, in 2009, Sato *et al.* generated 3D intestinal organoids from a single leucine-repeat containing G-protein coupled receptor 5 (Lgr5)-expressing adult intestinal

stem cell (34). These organoids were able to self-organize and differentiate into crypt-villi structures without the presence of mesenchyme. This was a landmark study that reported 3D organoid culture from a single stem cell and set the stage for subsequent organoid development from other systems such as stomach, liver, pancreas, retina, lung, and kidney.

Even within the hepatology community, differentiation of hepatocytes on floating collagen gels occurred in 1975 (35) and 3D culture of hepatocyte spheroids was quite prevalent in the 1980's and 1990's (36). This work included development of multicellular spheroids containing distinct cell types that were admixed and self-organized in 3D (37). Studies at that time also included work to transplant spheroid aggregates *in vivo* (38) and work on bioartificial liver devices using hepatocyte spheroids (39). In the early 2000's, the LaRusso group developed 3D culture systems consisting of bile duct units that generated cholangiocyte cystic structures *in vitro* for studies related to polycystic liver disease (40, 41). In fact, as others have pointed out, even the term "organoid" itself initially became popular in the 1970's in the context of developmental biology (42). In the past decade, however, there has been a parabolic explosion of interest in, and novel applications of, organoids (Figure 1). In particular, they are used as a method to model human development and disease *in vitro* (often in a patient-specific manner), to predict individualized responses to pharmacotherapy, to better simulate the *in vivo* environment, and to repair or replace damaged or diseased tissues (through cell-based therapy and / or bioengineering). While the technology seems newly emergent, it is more likely a natural extension of this prior work, now reaching its exponential phase.

Organoids, in the modern context, can be defined as 3D structures, grown *in vitro* from stem / progenitor cells or primary tissue, that self-organize in an organotypic manner through spatio-temporal patterning that may recapitulate developmental stages (e.g., stem cell-derived organoids) and/or resembles counterpart tissues *in vivo* (e.g. primary organoids). Organoid systems have been generated from human tissue or iPSC to resemble and model diverse organs such as liver, lung, kidney, heart, brain, and gut. The advantages and limitations of these 3D culture systems are summarized in Table1.

Within hepatology, there has been a vast proliferation of 3D culture systems, referred to as organoids, using various terminologies (e.g., hepatic, biliary, and pancreatic organoids; liver organoids; cholangiocyte organoids; cholangioids; ductal organoids; liver buds; etc.). This has led to some confusion and lack of precision in the scientific literature. In order to attempt to address

this issue, the HPB Organoid Consortium recently published a consensus document aiming to define a common nomenclature (43). This document defines an organoid more broadly, as follows: "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*." The group further subclassifies organoids into three broad categories, including: 1) Epithelial Organoids – containing a single germ layer from a single organ; 2) Multi-Tissue Organoids – containing multiple germ layers from a single organ; and 3) Multi-Organ Organoids – containing cell types from multiple organs. They go on to define how organoids can be further subclassified based on cell type of origin (e.g., pancreatic ductal organoid, intrahepatic cholangiocyte organoid, etc.). While this broadens the definition of an organoid, hopefully this new framework can provide some common understanding of the multitude of systems and procedures in use today. However, this classification may be less helpful for regenerative medicine applications which ultimately depend upon the functional properties of the organoids, rather than tissue of origin. Therefore, it may be necessary in the future to adopt additional nomenclature systems that better represent functional characteristics of the organoids.

Hepatocyte Organoids

The liver is a vital visceral organ with diverse, essential functions including basic metabolism, drug detoxification, clotting factor production, bile secretion, and protein synthesis. The liver parenchyma is composed of epithelial cells (hepatocytes and cholangiocytes) along with stromal, endothelial, and mesenchymal cells. Hepatocytes represent 95% of the liver parenchyma and are responsible for the lion's share of the liver's metabolic and synthetic functions. Recent advances in hepatocyte organoid technology are providing powerful platforms to generate new models to study development, physiological tissue homeostasis, and pathological transformation, which otherwise are intricate and difficult processes to study in humans. Below we discuss the development and biomedical applications of hepatocyte organoids as a tool for regenerative medicine.

During development, liver embryonic progenitor cells (hepatoblasts) are generated from the foregut endoderm in response to various signaling factors from the mesenchyme to form the liver bud (44). These bipotent AFP/KRT19+ liver buds can then give rise to the hepatocytes and

cholangiocytes depending on the exposure to the local signals (45). The hepatocytes also have a remarkable regenerative capacity *in vivo*, a property that explains the efficacy of surgical regenerative therapies such as hepatic resection and living donor liver transplantation. Despite this robust regenerative capacity *in vivo*, expanding liver cells *in vitro* has remained a significant challenge, one that hepatic organoids have now at least partially addressed. In 2001, early examples of liver organoids were generated when isolated adult rat hepatocytes and other hepatic cells were cultured in collagen-coated roller bottles leading to the formation of tissues resembling features of hepatic architecture (46). These organoids retained the structure and function of the hepatocyte epithelium, however the cultures survived only for a short period of time. Various iterations were attempted by different groups over time with similar limitations. In breakthrough studies in 2013, Huch *et al.* established the self-renewing, genetically stable, long-term expansion of adult murine-derived liver organoid cultures (47). This was achieved by combining Matrigel with hepatocyte growth factor (HGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and R-Spondin (RSPO1) with the isolated liver cells. Even single LGR5+ve cells had the ability to self-organize into 3D structure and differentiate into functional hepatocyte-like cells. Further optimization of the culture medium with the modulation of cAMP and TGFβ-signaling allowed the expansion of adult human liver cells as organoids (48). This system provided the first proof-of-principle for the regenerative potential of adult stem cells; however, the resulting hepatocytes still demonstrated some limitations in terms of function and maturity compared to their native counterparts. To address this challenge, the Nusse and Clevers labs recently built on this knowledge and developed novel systems for the long-term culture of primary mouse and human hepatocytes in the form of of hepatocyte organoids (49, 50). Importantly, the resulting organoids illustrated improved engraftment and regenerative capacity compared to previous systems. However, most of the work done was in mouse while the human hepatocyte organoids developed from fetal and adult tissue demonstrated differences in growth with adult hepatocytes showing limited expansion potential (49, 50). This might pose challenges with regard to access to tissue, upscaling, and manufacturing compared to adult stem cells.

An alternative approach to optimise engraftment is the transplant of lab grown whole liver tissue. The biggest challenge with this strategy is ensuring that the transplanted tissue will achieve adequate vascularization. To achieve this, in a different system, Takebe *et al.* employed human iPSC-derived hepatocytes with mesenchymal stem cells MSCs and umbilical cord cells (HUVECs)

to obtain embryonic liver bud organoids (51). These liver buds formed mature functional hepatic tissue upon transplantation into mouse. Moreover, the engrafted cells also secreted albumin as well as regenerated and rescued murine models of drug-induced liver failure. As a further optimization of the system, Takebe also obtained three cell types (hepatic endoderm, liver endothelium, and septum transversum mesenchyme) from the iPSC to form the liver buds, thereby avoiding the use of postnatal tissue progenitors (52). By modifying these culture protocols, several groups have now established liver models from different species such rats and dogs (53-55). Moreover, patient-derived organoid cultures have also been exploited to create disease models for Alagille syndrome (48), alpha-1-antitrypisn (A1AT) deficiency (48), polycystic liver disease (56), as well as cancer (57).

Even with all the above-mentioned multicellular organoids, none had contained the biliary structures that are essential to drain cytotoxic bile from the hepatic tissue. Several groups have tried to address this issue using complementary approaches. Guan *et al.* utilized iPSC-derived hepatic organoids surrounded by cholangiocyte duct-like structures (58). These organoids, when dissociated into single cells and replated in matrigel, maintained their proliferative and regenerative capacity. Wu *et al.* also established hepatic organoids from pluripotent stem cells that contained hepatobiliary structures. When transplanted in immune-deficient mice, these organoids survived for more than 8 weeks (59). Another study demonstrated the ability of Epithelial Cell Adhesion Molecule (EpCAM)-positive cells, but not EpCAM-negative cells, to form hepatic organoids as well as maintain the fidelity to be differentiated into functional hepatocytes (60). Recently Tanimizu N *et al.* developed a hepatobiliary tubular organoid system by combining hepatocytes and EpCAM-positive cholangiocytes in a mixture of collagen I and Matrigel culture (61). Under these conditions, hepatocytes established tight junctions with the cholangiocytes which themselves formed a tubular network. Hepatocytes were functional in this organoid system based on the secretion of metabolites into the canaliculi, which were then transported into the biliary tubular structures. Moreover, hepatocytes also acquired and maintained metabolic functions like albumin secretion cytochrome P450 activities. Thus, in this study, the authors were able to establish functional liver tissue incorporating a biliary drainage system *ex vivo* (61).

The above systems provide multicellular organoids containing biliary and hepatic structure; however, they did not fully recapitulate the different stages of hepatobiliary development, rendering them somewhat limited for developmental studies. To overcome this

issue, Koike *et al.* were able to model early endoderm morphogenesis using a mixture of foregut and midgut organoids (62). These organoids spontaneously organized into complex structures incorporating liver, pancreas, intestinal, and biliary progenitors through a process of invagination and branching that mimicked early development of the extrahepatic biliary tree at the foregut-midgut boundary. This system could provide precious insights into liver development and set the stage for developing functional 'mini-liver' units compatible with transplantation *in vitro*.

Cholangiocyte Organoids:

It can be argued that the biliary system is an even more attractive target for regenerative therapeutics for the liver since the biliary epithelium demonstrates a remarkable regeneration potential, is broadly distributed throughout the liver, and is accessible in a minimally invasive manner with endoscopic retrograde cholangiopancreatography (ERCP). Biliary epithelial cells (cholangiocytes) protect the liver and the surrounding tissues from the cytotoxic effects of bile (63). Furthermore, they modify bile composition by regulating the content of electrolytes, water, and bile acids (64) and deliver the contents to the small intestine. Although cholangiocytes represent less than 5% of the total liver parenchyma (65), disorders of the bile ducts (cholangiopathies) account for 25-30% of adult (66) and 70% of pediatric liver transplantation (64). This creates a pressing need for the development of therapeutic alternatives, such as cellular therapies. Historically, difficulties in culturing *bona fide* cholangiocytes *in vitro* have hampered their application in regenerative medicine for cholangiopathies. The development of cholangiocyte organoid systems has addressed this challenge and rapidly transformed the field. We summarize these advances and outline the main systems for culturing cholangiocyte organoids (COs) systems which can be derived from both healthy and diseased individuals and can be generated from a variety of sources including stem cells, primary tissue, or body fluids, such as bile (Figure 2B).

iPSC-based Systems: iPS-derived cholangiocytes were developed to address challenges in propagating primary cholangiocytes in 2D culture. To achieve this goal, the first cholangiocyte organoid platforms developed were based on iPSC which can be derived from readily available samples, such as skin fibroblasts or blood cells. To achieve differentiation of iPSC into cholangiocytes, multiple groups (56, 67-71) developed platforms recapitulating key stages of bile duct development. The common denominator in these different protocols was the differentiation

of iPSC to hepatoblast-like cells (which can give rise to both cholangiocytes or hepatocytes), and the subsequent commitment of these hepatoblasts to the biliary lineage. Developmentally, this lineage commitment process gives rise to a monolayer of cholangiocyte progenitors surrounding the portal mesenchyme (72), which then remodels into 3 dimensional ducts driven by Jagged1-Notch2 interaction between the ductal plate and the portal vein mesenchyme. To reproduce this process *in vitro*, cholangiocyte progenitors or hepatoblasts were cultured in 3D hydrogels, giving rise to the first cholangiocyte organoids. Matrigel is the most widely used hydrogel; however, gels more closely resembling the composition of the developing liver ECM such as laminin 411 and 511, have also been exploited with encouraging results (73).

Different strategies have been employed to achieve this *in vitro* equivalent of ductal plate remodeling; these include monocellular and multicellular approaches, each of which has its own advantages and limitations. In multicellular systems cholangiocyte commitment of hepatoblasts is enhanced by the interaction with OP9 cells, an irradiated stromal cell line expressing Notch ligand which recapitulatees the crosstalk with the periportal mesenchyme (70, 71). The resulting cells show expression of key cholangiocyte markers, such as CFTR, biliary functions, including flow-sensing primary cilia, and engraftment into immunocompromised mice (70). However, the paracrine signals between cell types in multicellular systems remain difficult to disentangle. On the contrary, monocellular systems are chemically defined as they are solely based on key biliary factors such as Notch, Wnt, TGFβ, and FGF (56, 67, 69, 71) reducing culture variability making monocellular systems more amenable for regenerative medicine applications. These cells express biliary markers such as CK7, CK19, CFTR and SOX9, present typical morphological features, including primary cilia, and respond to secretory stimuli (secretin and somatostatin). Remodeling in these platforms is thought to be initiated by the Jagged1-Notch2 interaction between cholangiocytes, using a mechanism known as lateral induction (74).

Considered collectively, iPSC derived cholangiocyte organoids provide a unique platform for generating biliary epithelial cells from virtually any patient and from a broad variety of starting material (e.g., skin, blood, urine), using minimally invasive procedures. This approach does not require access to primary tissue; the resulting cells recapitulate key cholangiocyte functions and can be used successfully for disease modelling and drug screening (56, 67-69, 71). Despite the presence of many features of mature cholangiocytes, they still are limited by incomplete

maturation, retention of some fetal characteristics, and the genetic instability that can be associated with iPSC (48).

Primary Tissue-Based Systems: The lessons learned from iPSC-derived cholangiocyte organoids were rapidly extended to primary tissue, giving rise to the first primary tissue-derived ductal organoids. These bile duct epithelial cells were grown using two main complementary platforms, based on canonical or non-canonical Wnt signaling. In the bile ducts, as in other organs (34, 47, 48), Wnt seems to be a master regulator of a mature vs. stem cell phenotype (45, 75).

The platforms based on canonical Wnt signaling propagate adult liver stem cells with a biliary phenotype (48), primarily through canonical Wnt and R-spondin1. Although the resulting cells express basic cholangiocyte markers, they are bipotent and able to differentiate toward both the hepatic and biliary lineage, acting as facultative stem cells in the liver (48). Conversely, this stemness comes at the cost of maturity. These adult stem cells or their differentiated progeny do not fully recapitulate the functions of mature cholangiocytes or hepatocytes *in vitro*. Derivation of such cells from humans is predominantly initiated from diseased livers, with reduced efficiency in healthy tissue. It is believed that the disease niche, which is, to some extent, recapitulated by canonical Wnt signaling *in vitro,* causes epigenetic remodeling of cholangiocytes, unlocking their stem cell potential (76).

Primary cholangiocytes grown in conditions based on non-canonical Wnt signaling give rise to mature primary cholangiocyte organoids (77, 78). These mature organoids can be cultured long term while maintaining genetic stability, expression of key mature biliary markers and cholangiocyte functions *in vitro*. Furthermore, they capture the diversity and plasticity of primary cholangiocytes (79). More specifically, bile duct epithelial cells are comprised of different subpopulations, which correspond to different anatomic compartments of the biliary tree (e.g., intrahepatic, extrahepatic, gallbladder). Primary organoids can be derived from any of these compartments (78) or from bile collected through ERCP (78). Importantly, the identity (intrahepatic, extrahepatic, gallbladder) of these cells *in vivo* and *in vitro* is not fixed, but is plastic and determined by the microenvironment, such as the composition of bile (79). Accordingly, primary cholangiocytes lose some of the characteristics of their subpopulation in culture and assume a common organoid identity; and vice versa, organoids exposed to different bile acids and concentrations of bile resume the *in vivo* identity corresponding to this microenvironment (79). It is possible that the two primary bile duct organoid platforms represent different ends of the

spectrum of the cells' plasticity between health and disease, and further research is required to clarify this point. However, despite originating from similar anatomical regions within the liver the two systems are not fully equivalent in terms of function and regeneration potential. To capture these differences for regenerative medicine, it is critical to classify cells based on their function and properties as they determine their potential clinical applications, rather than their anatomical origin. Therefore, for the purpose of this review, primary tissue-derived ductal organoids are classified as either adult stem cell organoids or mature cholangiocyte organoids. In summary, one of the main benefits of primary organoid systems is their genetic stability, maturity, and ease of propagation compared to iPSC-derived cholangiocyte organoids (80). However, they require access to primary tissue which may not always be possible, especially in rare conditions with limited numbers of patients.

Disease Modelling: Many types of cholangiocyte organoids have been derived from patients and used to recapitulate key aspects of biliary diseases *in vitro*. These diseases include infantile monogenic cholangiopathies, such as cystic fibrosis, Alagille syndrome and polycystic liver and kidney disease (56, 71) as well as adult and pediatric multifactorial biliary disorders such as primary sclerosing cholangitis (PSC) (81), biliary atresia (82), and cholangiocarcinoma (57). Importantly, iPS-derived cholangiocyte organoid disease models have been successfully used to screen and repurpose drugs for cystic fibrosis liver disease (56, 71), showing the potential of these models for developing new therapeutics. However, recent studies have shown that cholangiocytes are plastic and even primary organoids lose some of their *in vitro* characteristics in culture, when removed from their native niche (79). Therefore, it is possible that re-introducing different aspects of the disease microenvironment in culture may be critical to further enhance the phenotype of patient cholangiocyte organoids *in vitro*.

Non-Parenchymal Organoids:

Organoids based on non-parenchymal liver cells have also emerged. Hepatic stellate cells (HSCs) are the main mesenchymal cells within the liver, which are quiescent at baseline and store vitamin A droplets. They contribute significantly to liver physiology and pathology, especially in the context of fibrogenesis. Exposure to toxins or infections activate HSCs resulting in increased deposition of ECM in liver (fibrosis), which can lead to cirrhosis and portal hypertension. *In vitro* culture of HSCs poses the issues of limited proliferation capacity in 2D, loss of the quiescent

phenotype in culture, and loss of key functional features. Koui *et al.* tackled these issues by generating iPSC-derived HSC progenitors that matured and expanded *in vitro* (83). Another recent study by Coll *et al.* established another protocol for iPSC-derived HSCs through mesodermal progenitors. These progenitors when exposed to retinol and palmitic acid, differentiated into HSCs (84). The resulting cells resembled primary HSCs based on transcriptomics, functional assays, and morphology. In addition, when co-cultured with HepaRG, these cells in the spheroid form stored Vitamin A as well as switched from quiescent to activated HSCs in response to hepatotoxic stimuli such as thioacetamide and acetaminophen. Therefore, these systems represent a new model for studying HSC activation, liver fibrosis and toxicity. This is especially exciting in the context of screening anti-fibrotic therapies. More recently, Ouchi *et al.* developed a protocol to generate an organoid model recapitulating steatohepatitis. These organoids were generated from iPSC via a foregut differentiation step and included hepatocytes, stellate and Kupffer-like cells from healthy or NAFLD/NASH patients (85). This system represents a step forward in recapitulating features of complex disease phenotypes, such as steatohepatitis, including progressive steatosis, inflammation, and fibrosis *in vitro*.

Organoids in Liver Regenerative Medicine:

Liver organoids represent a paradigm shift in that they simultaneously provide a platform for a variety of both clinical and research use cases (Figure 3A). In the research realm, they are powerful systems for disease modelling and mechanistic studies on developmental processes, self-organization, cell-cell interactions and pathway perturbation, using tools such as genetic engineering and omics technologies. In the clinical sphere, liver organoids provide a platform which can be used for drug discovery and personalized medicine, and, importantly, a cell source for regenerative medicine-based therapeutics. Human liver organoids can be adapted cell-based therapies and/or for tissue engineering purposes. In this paradigm, autologous patient-derived organoids may eventually be combined with bio-scaffolds and regenerative biological factors to generate custom tissue engineered grafts for return to the patient (Figure 4A and B).

Although regenerative medicine for liver diseases has been explored as an alternative to transplantation for many decades, several challenges remain. Cells used for transplantation must meet certain standards to provide a viable therapeutic strategy. First, transplanted cells need to be highly functional. This is critical to restore organ function and survive in hostile environments,

such as in the presence of bile. Second, for systemic effects (e.g., secretion of A1AT in circulating blood) they need to engraft, survive long-term, and integrate in the host vasculature. Third, cellular therapies are not suitable when reconstructive surgery is required (e.g., in biliary reconstruction). Finally, to advance from proof-of-principle to clinical applications, they need to be compatible with large-scale expansion, and exhibit an acceptable safety profile, which includes genetic stability with no carcinogenic potential. The onset of organoid technology has addressed several of these challenges by providing functional, genetically stable, and highly proliferative cells (50, 77, 79), with a capacity to generate complex bioengineered tissues (77, 86) and to integrate into the host vasculature (51). Here, we provide an overview of the different liver organoid platforms developed, summarize both their advantages and limitations, and outline their applications for regenerative medicine (Table 2).

Hepatocellular Organoids: Broadly, 3 different types of hepatocellular organoids have been successfully transplanted in animal livers. IPSC-derived organoids (51, 52, 59, 60) adult stem cell-derived organoids (48) and primary hepatocyte/hepatoblast organoids (49, 50). In most studies, the cells were administered primarily through injection in the spleen (49, 50, 59, 60) or via ectopic transplantation (51, 52). In all cases, transplantation resulted in cell engraftment and function as evidenced by the detection of human liver markers (e.g., human albumin secretion) (48-52, 59, 60). Importantly, each of these systems has different advantages and limitations, which render them optimally suited for different applications. IPSC-based platforms are limited by concerns with regards to genetic stability and differentiation efficiency (87). The opposite applies to hepatocyte and adult stem cell-derived organoids, as primary cells they are less limited by genetic instability; however, they are restricted by access to primary tissue (49, 50). Adult stem cells can theoretically regenerate both the biliary and hepatic lineage and therefore they are more suited for diseases affecting both compartments of the liver (48). Primary hepatocyte organoids show higher function but remain committed to the hepatic lineage and are more suitable for diseases affecting predominantly hepatocytes (49, 50). Overall, in murine models, studies have been promising (48-50) but whether these findings can be translated to humans is yet to be demonstrated. This could theoretically be achieved by providing a scaffold to create an optimal niche that can support the homing, proliferation, and differentiation of the required amount of organoids.

Multicellular liver organoids: End-stage organ damage may render the liver microenvironment hostile and unsuitable for the transplanted cells to engraft. Furthermore, the damage to the liver tissue may extend to multiple cell types beyond hepatocytes. Therefore, hepatocyte transplantation may not be adequate to fully restore the liver function. To address these challenges, ectopic transplantation of multicellular organoids, containing a biliary system, may be required. These 'mini-livers' have been primarily transplanted ectopically in the cranium, in the mesentery or under the splenic capsule rescuing animal models of drug-induced liver failure (51, 52). So far, only iPSC-based multicellular organoids have been developed, which exhibit similar advantages and limitations to iPSC-derived hepatocyte organoids. Specifically, iPSC-derived multicellular organoids provide the advantage of not being limited by access to primary tissue, as iPSC can be easily generated from almost any tissue, which can be obtained through minimally invasive procedures (e.g., skin, blood, urine); however, they are restricted by concerns with regards to genetic stability and differentiation efficiency and maturity (87). Importantly, these organoids form independent mini liver units with functional vessels, which spontaneously integrate to the host's vasculature, rendering them suitable for ectopic transplantation. This overcomes the challenge of engraftment in a damaged liver with a hostile microenvironment. However, functional liver units also require biliary drainage. This might preclude ectopic transplantation in remote locations that provide no access to the biliary tree or intestine. Multicellular organoids with a biliary system that can integrate to the host biliary tree has not been demonstrated so far.

Cholangiocyte Organoids: The same 3 types of organoids used to regenerate the hepatocellular parenchyma have been used to regenerate bile ducts (iPSC-derived, adult stem cell, and primary tissue) (48, 70, 79). Organoids have been administered both intrasplenically (48, 70) and through the bile ducts via retrograde injection (79). However, in contrast to hepatocellular organoids, iPSC and adult stem cell-derived cholangiocyte organoids have only been shown to engraft but not to rescue animal models of biliary injury (48, 70). It is possible that this reflects the less mature state of the cells at the time of transplantation. Indeed, lack of maturation translates to reduced function (i.e., reduced bile resistance, increased cell death post transplantation, and reduced cell engraftment). Conversely, primary cholangiocyte organoids have been shown to regenerate up to 50% of the biliary tree following injury in mice (79). These organoids have also been successfully transplanted in human livers perfused *ex-situ*, rescuing ischemic cholangiopathy

(79). Importantly, this was the first demonstration of the efficacy of regenerative medicine using organoids in a human organ.

In cases of extensive injury, intact tissue is required for use in surgical reconstruction. This challenge is particularly evident in the extrahepatic biliary tree in the context of biliary atresia (BA). BA is a fibro-obliterative disorder affecting the common bile duct, which is replaced by fibrotic tissue (88). BA is the leading cause for liver transplantation in children (89). To address this challenge, primary cholangiocyte organoids have been combined with densified collagen scaffolds to generate bioengineered human bile ducts. These constructs were successfully transplanted into immunocompromised mice, providing one of the first proof-of-principle applications of organoids in regenerative medicine (77).

Advantages and Limitations of Organoids for Regenerative Medicine:

Advantages: The last decade has seen a substantial advancement in the development and use of organoid systems for regenerative medicine, enabling organoids to address challenges and provide advantages over 2D culture. First, organoid culture allows the propagation of highly functional cells. More specifically, iPSC-derived organoids show higher maturity and function compared to their 2D counterparts for both hepatocytes (51, 90) and cholangiocytes (49, 56, 70). Furthermore, both primary hepatocytes (49, 50) and cholangiocytes (77) have been propagated as organoids retaining most of their original function and properties. Maturity and enhanced function provide organoids with the capacity to engraft, regenerate the liver or bile ducts, and rescue animal models of hepatobiliary injury(49, 50, 77, 79). Organoid technology with genetic stability and highly expandable properties could be a future alternative treatment strategy to organ transplantation. Preclinical studies with mouse models have shown promising results in which mouse intestinal organoids were engrafted into a damaged colon where they formed functional crypt units (91). Similar to intestine, mouse adult liver organoids also rescued liver failure and prolonged survival upon transplantation into fumarylacetoacetate-hydrolase (FAH) mutant mice, a mouse model for tyrosinemia type I liver disease (47). Even stem cell derived liver organoids restored hepatic functions and rescued acute liver failure in mice (48). More recently the feasibility of treating bile duct disorders with cholangiocyte organoids has been in proof-of-principle studies using human liver perfused *ex-situ* (79).

Importantly, primary organoids demonstrate a unique expansion potential compared to iPSC, which makes them suitable for large scale expansion and manufacturing. Moreover, organoids allow the generation of complex autonomous mini liver structures with their own vascular network, which can integrate into the host vasculature and be transplanted in any vascular niche outside the liver (51, 52). Equally, bioengineered bile ducts get rapidly vascularized *in vivo* following scaffold remodelling (77) . Therefore, organoids provide an optimal source of cells for tissue engineering. Considered collectively, these advantages address many of the existing challenges for regenerative medicine.

Limitations: Despite the speed at which the organoid and regenerative medicine fields are advancing, multiple challenges to clinical translation remain. In this section, we describe these challenges and how they could be addressed in the future. Organoids are cultured in 3D conditions, which are not compatible with current, automated, large-scale manufacturing platforms. Nonetheless, new robotic systems being developed show great promise for overcoming this limitation (92). Alternative culture systems, such as spinning flask bioreactors (92, 93) also show promise in addressing large-scale manufacturing hurdles (92, 94).

Furthermore, organoid culture is based on non-chemically defined hydrogels, such as Matrigel. Despite its advantages, Matrigel is a complex hydrogel produced from a mouse Englebreth-Holm-Swarm sarcoma cell line posing potential risks for tumorigenesis and immune reaction due to the presence of mouse contaminants and can be characterised by high batch-to-batch variability. To address these limitations, fully defined hydrogels based on biological or synthetic polymers which are compatible with Good Manufacturing Practice (GMP) are being developed (95, 96). Biological hydrogels based on ECM macromolecules such as collagen or laminin, are cheap and widely available with promising results for supporting hepatocyte differentiation (97). However, single component hydrogels fail to capture the complexity of the native ECM and do not provide the full spectrum of biochemical cues required by cells. Conversely, hydrogels derived from decellularized tissue largely preserve the composition and biochemical cues present in native tissue and have been shown to support the growth of 3D biliary and liver organoids (98, 99). Nevertheless, decellularized ECM hydrogels are limited by donor availability and they are not chemically defined. This renders modifying the composition and mechanical properties of the gel more difficult and poses challenges for mechanistic studies. Hydrogels based on synthetic polymers, such as, poly-ethylene-glycol (PEG) and polyvinyl acetate

(PVA), offer greater control over the mechanical properties of the gel, as they are not limited to ECM proteins at the expense of using physiological cues. To combine the benefits of synthetic and biological gels, 'hybrid gels' have been developed incorporating distinct biochemical cues, such as collagen, fibronectin, or laminin in PEG-based hydrogels to support hepatic differentiation of iPS cells (100). However, these cells still showed limited liver functionality suggesting the lack of critical biochemical cues/growth factors. One of the drawbacks of developing such hybrid gels is that screening multiple parameters to identify the optimal combination for different cells can be time-consuming and cost prohibitive. Although recent developments have enabled the synthesis of more affordable hydrogels with increasingly complex functionalization methodologies, enabling the stable release of growth factors in culture (101), Matrigel still remains the most cost-effective and versatile solution for organoid culture. Extensive characterization of the actual risks posed by Matrigel could result in approving the cells grown in this matrix for clinical use. There are multiple examples that provide precedent for this approach, including the use of animal products, such as porcine heart valves and intestinal mucosae and the use of cells grown in serum in clinical trials (102-104).

Although the generation of mouse-sized bioengineered bile ducts provided proof-of-principle for the use of organoids in tissue engineering, upscaling to human sized constructs may require scaffolds recapitulating the complex architecture, mechanical properties, and different cell types of the native tissue. To address this challenge, the ECM of the native liver could be exploited using the process of decellularization. Decellularization removes all cellular components but preserves all the tissue-specific structural and functional components of the ECM (63). This results in a biocompatible and bio-degradable scaffold with intact vascular and biliary networks (105, 106). Decellularized human liver scaffolds can then be repopulated with functional liver cells (105, 106). Although cholangiocyte organoids (107, 108) have also been used to repopulate small flat patches of decellularized extrahepatic ducts, the potential of this approach for tissue repair or regenerative medicine has not been demonstrated. Challenges related to vascularization and upscaling this technology to human size still remain to be addressed. In addition, decellularization is limited by access to human tissue. The use of animal (e.g., porcine) tissue; synthetic scaffolds with an embedded vascular network (109), or 3D printed bioengineered tissue (110) may eventually overcome this issue.

The use of autologous organoids for regenerative medicine may not be practical for all scenarios for a variety of reasons. First, organoid derivation is a lengthy process and therefore may not be possible in cases of patients with acute liver failure who will require off-the-shelf regenerative medicine products. Second, autologous patient-derived primary organoids may remain affected by the disease and exhibit a reduced capacity to regenerate organs. Third, in some cases, access to primary tissue may not be possible (e.g., cholangiocytes in vanishing bile duct syndrome). These challenges could potentially be addressed by genetically engineering the organoids to avoid recognition from the immune system, e.g., by performing HLA knockout (111) or over-expression of the immune checkpoint PD-L1 (112). Alternatively, the creation of organoid biobanks, which will allow donor / recipient matching, similar to the immuno-compatibility approach currently used for liver transplantation, could address this issue.

The use of organoids in clinical trials remains extremely limited, with the only example coming from the salivary gland field. One of the biggest questions remains the regulatory classification of organoids in the spectrum between minimally (e.g., MSCs) or highly (e.g., iPSC) manipulated cells. Primary organoids do not undergo reprograming, which defines the 'highly manipulated' state of iPSC, consequently, iPS cells inherently carry a higher risk of tumorigenesis than primary organoids. Conversely, primary organoids demonstrate higher genetic stability have a lower risk of tumorigenesis, at least theoretically. However, they are still grown in a non-chemically defined hydrogel, which has prevented their use in clinical trials so far. Overcoming this limitation will set a regulatory precedent as Matrigel is used by multiple *in vitro* cell platforms beyond organoids and it will significantly expedite future trials. Therefore, the efficacy and safety, including the tumorigenic potential, of primary or iPSC organoids for regenerative medicine needs to be thoroughly assessed in long-term longitudinal large animal studies, before proceeding to first-in-human studies.

Conclusion:

We find ourselves at a unique point in history in which a variety of rapid technological advances are converging simultaneously and at an accelerating rate. Breakthrough advances in 3D culture of liver organoids and regenerative medicine approaches hold enormous promise for improving the lives of patients with advanced liver disease. In this review, we have attempted to define the current state of regenerative hepatology by first reviewing the historical precedents that

empowered development of the field and then outlining the current state-of-the-art science. We have reviewed basic principles of regenerative medicine applied to the liver, discussed both hepatocellular and biliary organoids in detail, and explored the advantages and limitations of organoid-based systems. The remaining challenges will include keeping pace with rapid technological change, while simultaneously translating the regenerative concepts and tools into bona fide regenerative therapeutics. Doing so is likely best accomplished through broad collaborations and multi-disciplinary teams including basic and translational scientists, bioengineers, hepatologists, hepatobiliary and transplant surgeons, and advanced endoscopists. Although many challenges remain, we expect to soon see dividends from the decades of work that lead us to this exciting moment. It is our hope that new regenerative hepatology service lines will become broadly available and eventually represent a paradigm shift in the management of patients with acute and chronic liver disease.

Acknowledgement: Figures created with BioRender.com

Features	2D	3D
Culture Microenvironment	**Advantages** ➢ Ideal for mimicking the barrier function of an epithelium between different compartments (e.g. Transwell, air liquid interphase) **Disadvantages** ➢ Usually simple matrices used (e.g. collagen) ➢ Cell to ECM interactions is limited to the cells' basal surface ➢ Less customizable matrix mechanical properties (e.g. stiffness)	**Advantages** ➢ Complex ECMs used ➢ Recapitulate microarchitecture of native tissue ➢ Lumen formation allows recapitulation of intra-luminal vs. extra-luminal space with active transfer between compartments ➢ The matrix can be manipulated to study the cells' response to the mechanical properties of their microenvironment (e.g. studying cirrhosis) **Disadvantages** ➢ Difficult to sample different compartments (organoid lumen vs. extraluminal space)
Cell properties	**Advantages** ➢ Can be grown in chemically defined and GMP-grade conditions compatible with regenerative medicine applications **Disadvantages** ➢ Reduced functionality when using the same cell type in 2D vs. 3D ➢ Cell-to-cell interaction is restricted to 2D ➢ Loss of polarity markers unless certain systems (e.g. Transwell) are used ➢ 2D systems do not support long-term expansion of most of the liver primary cell types	**Advantages** ➢ Increased functionality compared to 2D, most likely due to the advantages in recapitulating the cells' physiological niche ➢ Higher level of maturity achieved for differentiating cells (e.g. iPS cells) ➢ Ideal for recapitulating the spatial relationship between different cell types ➢ Cells can self-organize in 3D mimicking their physiological niche ➢ Proven potential for regenerative medicine and bioengineering applications ➢ Allows long term culture and expansion of primary cells **Disadvantages** ➢ Complex ECMs are often non-chemically defined, not GMP-compliant and therefore may not be compatible with regenerative medicine applications
Clinical translation	**Advantages** ➢ Cost-effective ➢ Quick growth in culture ➢ Exponential growth	**Advantages** ➢ Allows long-term expansion of primary cells, which are minimally

	➢ Can be cultured using robotic systems ➢ Compatible with large-scale manufacturing ➢ Homogeneous **Disadvantages** ➢ Not compatible with expansion of most primary adult liver cell types ➢ Highly manipulated cells (e.g. iPS cells) need to be used to replace native tissue, which poses regulatory barriers	manipulated posing less barriers to clinical translation **Disadvantages** ➢ Expensive ➢ Time and labor-intensive culture ➢ Few robotic systems available ➢ Less compatible with large scale expansion and manufacturing ➢ More heterogenous systems rendering quality control more challenging
Technical aspects	**Advantages** ➢ Technically easier to culture ➢ Most assays are optimized for 2D culture making it easier to characterize cells. ➢ Easier to extract cells for live assays compared to 3D ➢ More homogeneous culture, compatible with characterization through bulk assays **Disadvantages** ➢ Cannot be used for studies on self-organization and morphogenesis ➢ Co-culture experiments are more challenging, as 2D culture limits the movement and interaction of cells compare to 3D	**Advantages** ➢ Optimal for studies in self-organization and morphogenesis. ➢ Ideal for multicellular culture and studies on cell-to-cell signaling and interactions **Disadvantages** ➢ Technically more challenging to culture, maintain and extract cells. ➢ Requires higher resolution techniques for characterization of multicellular systems – e.g. single cell RNA sequencing

Table 1. Comparison of 2D and 3D culture systems.

Cell type for Organoid Culture	Route for delivery	Organism	Outcome	Reference
Immortalized human hepatocytes, hepatic stellate cells and endothelial cells	Renal subcapsular space, intra-abdominal	BALB/c Nude Mice	Increased Albumin, connexin 26 and 32, HNF4 α and glucose-6-phosphatase expression at 8 weeks after transplantation	(113)
Mouse single Lgr5+ve stem cells	Intrasplenic Injection	Fah–/– Rag2–/– Il2rg–/– mice	0.1-1% engraftment in the liver parenchyma up to 3 months after transplantation. Increased survival compared to non-transplanted controls	(47)
Human Lgr5+ve liver stem cells	Intrasplenic injection	Balb/c nude mice with CCl4-retrorsine to induce acute liver damage	Limited engraftment and albumin secretion up to 40 days	(48)
Human iPSC-derived cholangiocytes	Transplantation of Matrigel plug into mammary fat pads	NOD-SCID-IL2rγ−/−; NSG	Formation of duct-like structures with cholangiocyte characteristics (e.g. cilia) within 6-8 weeks of transplantation	(71)
Human iPSC-derived liver cells	Renal subcapsular space	Alb-TRECK/SCID mice with acute liver failure induced by diphtheria toxin	70% survival at 20 days after transplantation compared to 30% for the controls (sham). Albumin secretion up to 7 days	(114)
Human and mouse primary hepatocytes	Intrasplenic injection	Fah−/− NOD Rag1−/− Il2rgnull mice	Engraftment, albumin secretion and CYP2E1 expression up to 90 days after injection	(50)
Mouse primary hepatocytes	Intrasplenic injection	Fah-/- mice	Up to 80% engraftment 100 days post transplantation. Albumin secretion and hepatocyte marker expression, (e.g. CYP2E1, GLT1).	(49)

Human iPSC-derived liver cells	Ectopic transplantation (cranial window and renal subcapsular space)	Immunodeficient mouse (NOD/SCID and TK-NOG with Ganciclovir administration for liver injury)	Rescue in mouse models of acute liver failure, increased survival over primary hepatocyte transplanted mice. Albumin and α1-antitrypsin synthesis, effective detoxification (e.g., NH_3 clearance) up to 60 days after transplantation.	(51, 52)
Primary human hepatocytes, fibroblasts, endothelial cells	Implantation into the into the mesenteric fat	FAH-/- NOD mice with NTBC administration to induce liver injury	Engraftment and albumin secretion up to 80 days after transplantation. Increased proliferation, metabolic activity (e.g., albumin, transferrin, α1-antitrypsin) and vascularization following chronic liver damage.	(115)
Human iPSC-derived hepatobiliary cells	Intrasplenic transplantation (in a subcapsular pocket)	NOD-SCID mice	Engraftment of biliary structures up to 8 weeks post transplantation. No formation of teratomas.	(59)
iPSC-derived EpCAM-positive endodermal cells	Intrasplenic injection	NOD.Cg-Prkdcscid Il2rgtm1WjI/SzJ mice treated with dimethylnitrosamine (DMN) for 14 days to induce acute liver damage	Limited engraftment and albumin expression up to 32 days after transplantation. Increased albumin production upon engraftment of cells 32 days after transplantation	(60)
Human iPSC-derived liver cells	Intrasplenic injection	Athymic nude rats (Crl:NIH-Foxn1rnu) injected with D-Galactosamine to induce acute liver failure	Rescue in mouse models of liver failure, increased survival over non transplanted mice.	(116)

			Albumin expression up to 14 days after transplantation	
Mouse liver progenitor and endothelial cells	Vascularised chambers created in the right and left groin of every animal filled with Matrigel and hydrogel. Organoids transplanted in 15ul Matrigel on both sides.	Fah−/−/Rag2−/−/Il2rg−/−	Limited engraftment and expression of hepatic markers (e.g., SOX9, HNF4a) up to 12 days after transplantation .Increase in host lymphatic vasculature	(117)
Human iPSC-derived liver cells	Portal vein injection	Immunocompromised rats with partial hepatectomy	Cell engraftment and proliferation (injected cells constitute up to 70% of the liver parenchyma 120 days after transplantation), rescue of partial hepatectomy model, decreased ductular reaction and albumin secretion up to 12 days after transplantation. No signs of ectopic engraftment.	(118)
Fetal liver-derived organoids and human-induced pluripotent stem cell (iPSC)-derived liver cells	Portal vein	Porcine	Safe transplantation in the target lobe of the liver with ligation of the ductus venosus	(119)
Primary human cholangiocytes	Collagen tubular scaffold to reconstruct the common bile duct	NOD.Cg-PrkdcscidIl2rgtm1Wjl (NSG) mice with extrahepatic biliary injury	Engraftment of the bioengineered bile duct and rescue of mouse model of extrahepatic bile duct injury (survival up to 104 days). Grafts remained patent,	(77)

			maintained biliary function (e.g., ALP activity) and were vascularized *in vivo*.	
Primary human cholangiocytes	Intraductal delivery in human and mouse bile ducts	Human liver grafts with ischaemic cholangiopathy maintained with *ex-situ* normothermic perfusion Immunodeficient NSG mice (NOD.Cg Prkdcscid Il2rgtm1Wjl/SzJ) with toxin-induced cholangiopathy	Human: 40-85% engraftment in the injected ducts and improvement of bile pH and choleresis rescuing human organ model of ischaemic bile duct injury. Mouse: 25-55% engraftment and resolution of liver biochemistry (e.g., ALP, bilirubin) rescuing mouse model of diffused intrahepatic cholangiopathy up to 3 months after transplantation.	(79)
Human iPSC-derived cholangiocytes	Intrasplenic injection	TK-NOG mice	Engraftment and expression of biliary markers (e.g. CK7, CK19 and acetylated tubulin) up to 6 weeks after transplantation	(70)
Human iPSC-derived posterior gut endoderm cells	Renal subcapsular space	Immunodeficient liver failure mouse model (Alb-TRECK/SCID)	Engraftment, expression of hepatic markers and increased survival up to 30 days after transplantation.	(120)

Table 2: Summary of the main studies using liver organoids for regenerative medicine.

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Figure 1

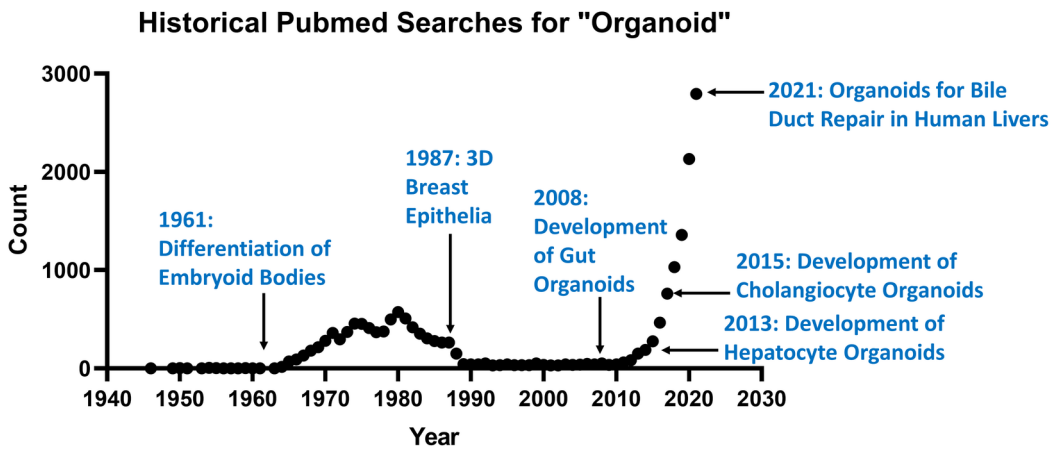

Figure 1. The rise of organoids in biomedical research. Graph showing the number of published manuscripts since 1940 found following a Pubmed search for the word ”organoid”. Landmark studies in the field are highlighted.

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Figure 2

A.

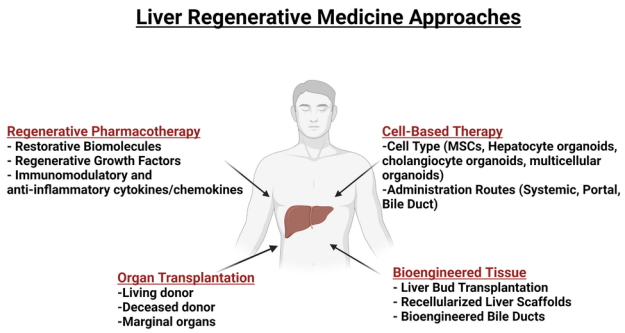

Figure 2A. Regenerative medicine in hepatology. Schematic outlining different regenerative medicine strategies for liver disease.

B.

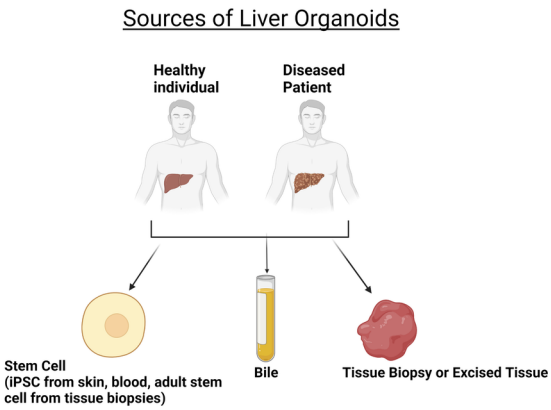

Figure 2B. Organoid sources. Organoids can be derived from multiple sources, including iPSCs, bodily fluids, e.g., bile, and primary tissue

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Figure 3

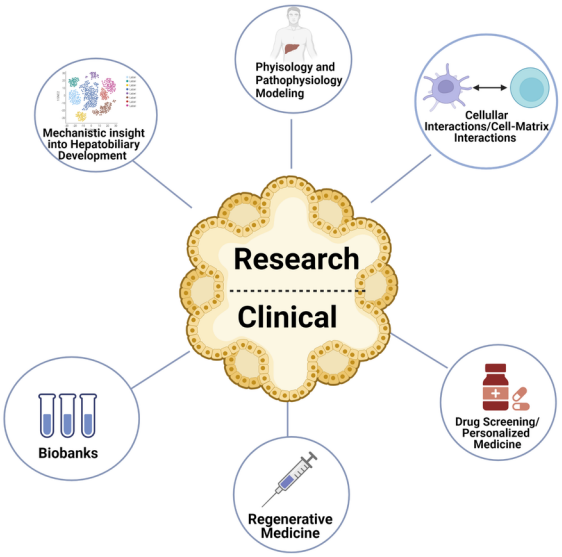

Figure 3. Organoid applications. Organoids have a broad spectrum of applications, ranging from basic research to clinical translation.

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Figure 4

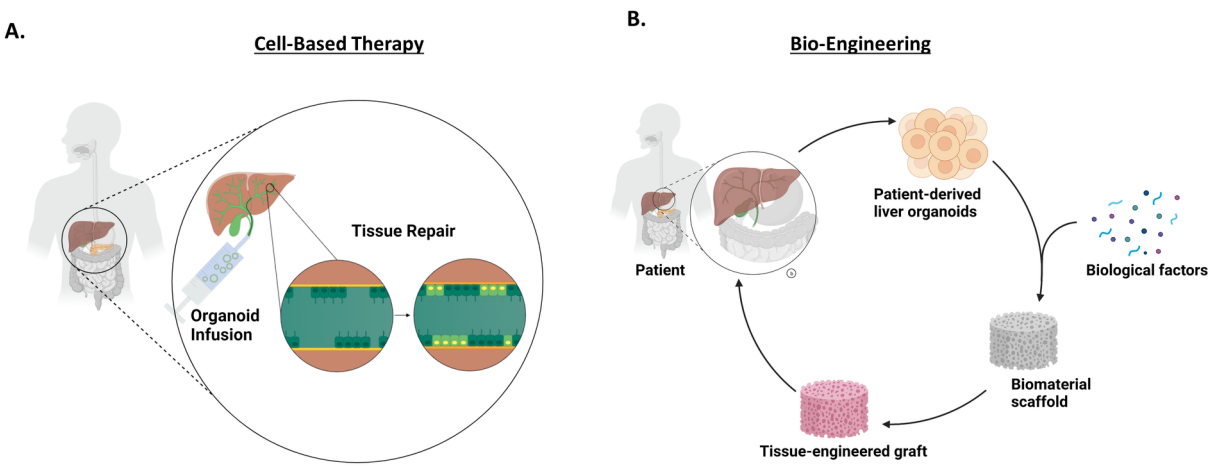

Figure 4. Organoid applications for regenerative medicine. **A-B**) Schematic summarizing applications of organoid technology for regenerative medicine, including cell-based therapy **(A)** and tissue engineering **(B)**.

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