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REVIEW
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Charting the Explicit Path: Translational Dynamics of Hepatic Bioengineering from Experimental Benchmarks to Practical Bedside Applications

Subathra Radhakrishnan[1], *Catherine Ann Martin*[1], *Geethanjali Dhayanithy*[2], *Josette Caroline Martin*[3], *Abinaya Chandrasekaran*[4], *Subbaraya Narayana Kalkura*[2], *and Mohamed Rela*[1,5*]

[1]National Foundation for Liver Research, India

[2]Crystal Growth Centre, Anna University, India.

[3]Department of Pathology, Sri Venkateshwara Medical College Hospital and Research Institute, India

[4]Department of Veterinary and Animal Sciences, University of Copenhagen, Denmark

[5]Dr. Rela Institute and Medical Centre, India.

Correspondence:
Mohamed Rela
mohamed.rela@relainstitute.com

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ABSTRACT

Liver transplantation is the only optimal method used for treating end-stage liver disease, originated in 1957 and was developed as a mainstream methodology over a span of two decades. Nevertheless, this procedure remains in a state of perpetual evolution, marked by ongoing advancements and adaptations. Moreover, the field of liver transplantation is still baffled by the non-availability of donor livers. Numerous scientific and technical innovations, both direct and tangential to liver transplantation, have emerged, contributing to its refinement, and augmenting the overall progress in this intricate domain. However, many of these findings have not yet been translated into clinical practice. Hepatic bioengineering has become a potential research model that physicians look up to as a treatment option for patients suffering from liver disorders. Research into therapeutic options like cell-based therapy, 3D tissue construction, bioengineering of the liver, and extracorporeal devices for patients who remain at the recipient juncture for a long duration is imperative. Yet, it is a difficult undertaking because the liver is a complex organ that performs several metabolic processes and biotransformation. Additionally, the organ requires continuous perfusion for the delivery of nutrients and oxygen as well as the elimination of waste. Myriad scientific groups are researching bioartificial livers, a supporting device incorporated with metabolically active liver cells to perform liver-specific functions. This review provides current developments in the field of liver regeneration in 2 & 3-dimensional environments and examines each of their pros and cons. It also provides the intersecting points that could be potentially used to overcome various lacunae in the liver transplantation field envisaged using bioengineering as a tool.

Highlights

- This review emphasizes regenerative medicine's vital role in treating ESLD, resisting organ scarcity and transplant waitlists.
- It sheds light on creating a non-antigenic, functional liver through reseeding parenchymal cells in acellular liver matrices from marginal livers and the importance of multicellular liver organoid seeding.
- Offering a comprehensive overview of researchers' creative and intellectual

endeavors, culminating in the development of the bioartificial liver, this review also highlights the necessity of an extracorporeal liver device.
- The review stresses the need for stringent transplantation guidelines, ethical considerations, and robust preclinical study validation.

Keywords: Liver Diseases • Cell-Based Therapy • 3D Constructs • Bioengineered Liver • Bioartificial Liver • Liver Tissue Engineering

Abbreviations: 3D: 3 Dimensional; 2D: 2 Dimensional; ESLD: End Stage Liver Disease; ECM: Extra Cellular Matrix; LT: Liver Transplantation; ALF: Acute Liver Failure; VEGF: Vascular Endothelial Growth Factor; BAL: Bio-Artificial Liver; ESCs: Embryonic Stem Cells; MSCs: Mesenchymal Stem Cells; iPSCs: Induced Pluripotent Stem Cells; TGF-β: Transforming Growth Factor; WNT: Wingless-Related Integration Site; IDE: Induce Definitive Endoderm; BMP: Bone Morphogenic Protein; FGF: Fibroblast Growth Factor; HGF: Hepatocyte Growth Factor; OSM: Oncostatin M; PLGA: Poly-Lactide-Co-Glycolide; pH: Potential of Hydrogen; PNIPAAm: Polyethylene Glycol (PEG)-Poly (N-isopropyl acrylamide); IPSC-HE: Human iPSCs Hepatic Endoderm; HUVECs: Human Umbilical Vein Endothelial cells; PEG: Polyethylene Glycol; HBTO: Hepatobiliary Tubular Organoids; FXR: Farnesoid X Receptor; HepLPCs: Liver Progenitor-like Cells from Human Primary Hepatocytes; Ali-BAL: Air-Liquid interactive Bioartificial Liver; MELD: Models for End-Stage Liver Disease; PBMCs: Peripheral Blood Mononuclear Cells; GMP: Good Manufacturing Practices; IND: Investigational New Drug; BLA: Biological License Application; NDA: New Drug Application; IRB: Institutional Review Board; CAD: Computer-Aided Design; FBS: Fetal Bovine Serum; GSK3: Glycogen Synthase Kinase 3; MTG: Monothioglycerol; DMSO: Dimethyl sulfoxide; HLC: Hepatocyte-like Cells; HNF4A: Hepatocyte Nuclear Factor 4 alpha FOXA2: Forkhead Box A2; ROCK: Rho-Associated Protein Kinase Inhibitor; PLACL: Poly (l-lacticacid)-co-Polyc(e-caprolactone); RAC1: Ras-related C3 botulinum toxin substrate 1; HFF-CM: Human Fetal Fibroblast-Conditioned Medium; SOX17: SRY-box Transcription Factor 17; CXCR4: C-X-C Motif Chemokine Receptor 4; ADA: Alginate Dialdehyde; CYP-P450: Cytochrome P450; SA: Sodium Alginate; PVA: Poly Vinyl Alcohol; hPCLS: Human Precision-cut Liver Slices (hPCLS); mRNA: Messenger RNA; PHH: Primary Human Hepatocyte; PU: Polyurethane; PANI: Polymer Polyaniline; SrO_2: Strontium Peroxide

INTRODUCTION

Chronic liver Failure or end-stage liver disease is the major cause of global health burden, with a 46 % increased mortality rate in the past three decades [1,2]. The global prevalence of cirrhosis has increased, and the common causative factors are alcohol, Non-Alcoholic Steatohepatitis (NASH), and viral Hepatitis. 2.95% of total deaths in India are because of Chronic liver diseases. In India, end-stage Liver diseases are the tenth most common cause of death reaching 264,193 or 3.00% of total deaths [3,4]. Hereditary hemochromatosis, Alpha-1-antitrypsin deficiency, and Wilson's disease are the major metabolic liver diseases prevailing in India. Such end-stage liver diseases require liver transplantation/ an alternate therapeutic approach, which aids in self-regeneration/restoration since the standard medications and surgical procedures [Liver Transplantation (LT)] were not sufficient.

Due to its inherent characteristics, the liver possesses a remarkable regenerative capacity. Following a sub-lethal injury, the human liver can undergo compensatory hypertrophy, characterized by the enlargement of hepatocytes, followed by hyperplasia. This intrinsic regenerative ability enables the restoration of parenchymal tissue [5-7]. This phenomenon has formed the basis of conservative management of acute liver failure, major liver resection for tumors, and living donor liver transplantation. Nevertheless, the patient needs significant support (when a considerable mass needs to be resected) for multiple functions during this period of regeneration as the liver is the body's metabolic powerhouse. In a distinct context, chronic liver injury results from varied etiologies, the Extracellular Matrix (ECM) of the liver itself has been disrupted because of the excessive deposition of ECM proteins [7]. Such cirrhotic livers which are accompanied by fibrosis also require an ECM remodeling to support liver regeneration or wound healing [8]. Even though liver transplantation (from live or brain-dead donors) saves lives in these scenarios, the clinical complications and life-long immunosuppression stress the importance of therapeutics with low risks and better organ restoration. Therefore, there is a huge need for auxiliary liver support in the form of cell therapy or extra corporeal devices encompassing organ bioengineering. This has been an in-focus work for the past three decades in the field of liver regenerative medicine.

Regenerative medicine is in its early stages of development, representing an emerging field in medical science to replace damaged tissues or organs. This can be achieved through the creation of a simulated environment using a 3D matrix or by employing cellular therapy. In 1999, the term "regenerative medicine" was coined by William Haseltine [9]. In the modern world, regenerative medicine holds a huge potential for medical research. Comprehensively, this branch of medical science comprises stem cells with self-renewability and good proliferation index, biomaterials with encapsulation for cell support and differentiation, prosthetics, bioengineered tissues, and artificial organs to restore the functionality of impaired organ systems [10].

The expedition, hepatic bioengineering started with simple differentiation of stem cells to hepatocytes on a tissue culture plate and was further extrapolated to form millions of cells in a bioreactor. Such cells displayed characteristic

polygonal morphology and possess hepatocyte-specific gene expression, but *in vivo*, they are partially functional. Administration of such therapeutic stem cells in cell-based therapies resulted in various complications including dissection of the hepatic artery, Tako-tsubo syndrome, cutaneous immunological disorder, hepatocellular carcinoma, and is also vulnerable to sepsis [11]. Besides, hepatocytes alone cannot perform the efficient functions of the liver, as they are discrete entities and do not form a tissue. Hence improved outcomes of such cell-based therapies are much needed as they result in short-term efficacy due to the loss of non-parenchymal cells and their signals.

In a normal liver, the ECM and the cells work together to maintain homeostasis in the system [12]. For the long-term efficacy of cell-based therapies a 3D functional liver construct that can house the hepatocyte population is required which can mimic the normal ECM. Similar constructs made with biocompatible molecules in the geometric form of the parent tissue and encapsulated with stem cells/differentiated cells transplanted to a damaged organ have been reported with clinical success. The major drawbacks of 3D tissue constructs are the absence of non-parenchymal cells and vascularization. The integrity and functional life of a biomimetic liver construct depend on the colonization and secretion of all the types of resident cells in the liver microenvironment. This dense cell-laden construct requires appropriate vascularization, in the absence of which hypoxic-induced necrosis occurs.

The third generation of bioengineered tissue places significant emphasis on the establishment of pre-vascular networks through the coordinated application of angiogenic factors, such as Vascular Endothelial Growth Factor (VEGF) and human endothelial cells. This approach enhances neovascularization *in vivo* following the implantation of the construct, as demonstrated by Asakawa et al. in 2010 [13]. Such advancements are pivotal in laying the foundation for the development of clinically relevant liver-like organs in laboratory settings.

This review begins by tracing the evolutionary trajectory of liver bioengineering, starting from cell-based therapies, progressing through three-dimensional constructs, organoids, and Organ decellularization followed by recellularization and culminating in the development of bioartificial liver technologies as the early stages of research and product development. Subsequently, it examines the clinical perspective, highlighting the potential implications of these advancements in the treatment of liver failure and discussing the validation of research findings. Altogether, this review conducts a comprehensive analysis of the journey from laboratory bench experimentation to bedside application in the scope of liver tissue engineering.

LITERATURE REVIEW

Understanding the Hepatic Differentiation of Stem Cells to Aid Developing *In-Vitro* Hepatic Differentiation Protocols

The evolution of artificial liver regeneration and its based therapy started with the understanding of hepatic development and differentiation *in vivo*. This understanding also plays a vital role in developing protocols for *in vitro* differentiation of stem cells into differentiated hepatic cells. The process of hepatocyte differentiation of stem cells is exclusively guided by the developmental cues observed during liver formation *in utero*. Consequently, each protocol designed for this purpose, whether directly or indirectly, aims to replicate the extrinsic signals present in the natural microenvironment of the liver. Embryologically, intrahepatic bile duct formation is initiated by the bipotential hepatic fate of specified endoderm cells (hepatoblasts) in the liver bud which migrate into the septum transversum and give rise to both hepatocytes and cholangiocytes depending on the microenvironment signal [14,15]. The understanding of events occurring at the cellular niche also plays a vital role in developing *in vitro* protocols for hepatocyte differentiation and liver morphogenesis. The various factors involved in the process of differentiation as discussed below and shown in Figure 1. *In vitro*, hepatic differentiation is achieved through molecular signal-induced genetic reprogramming of unspecialized cells like Embryonic Stem Cells (ESCs) / Pluripotent Stem Cells (PSCs) / Induced Pluripotent Stem Cells (iPSCs)/ adult Mesenchymal Stem Cells (MSCs). These ESCs/ PSCs/ iPSCs acquire cellular traits of hepatic lineage through i) cytokine-based differentiation [16-21] ii) epigenetic-based differentiation [22-24] iii) enhanced differentiation through co-culture with non-parenchymal cells [25,26] and iv) small molecule driven hepatocyte differentiation [27-30].

In mimicking *in vivo* embryonic liver development, *in vitro* differentiation also requires a definitive endoderm intermediate. Activin A is a member of the Transforming Growth Factor Beta (TGF-beta) superfamily involved in endoderm induction either individually or in combination with sodium butyrate, WNT 3a, and Induce Definitive Endoderm 1 & 2 (IDE1&2) [16-21]. Efficient generation of hepatocyte-like cells can be achieved from definitive endoderm through Wnt regulators, BMP4, FGF2, and FGF8 [27,31]. Interleukin 6 family member, Oncostatin M (OSM), and paracrine factor Hepatocyte Growth Factor (HGF) are the prime molecular signals for hepatic maturation. Di-

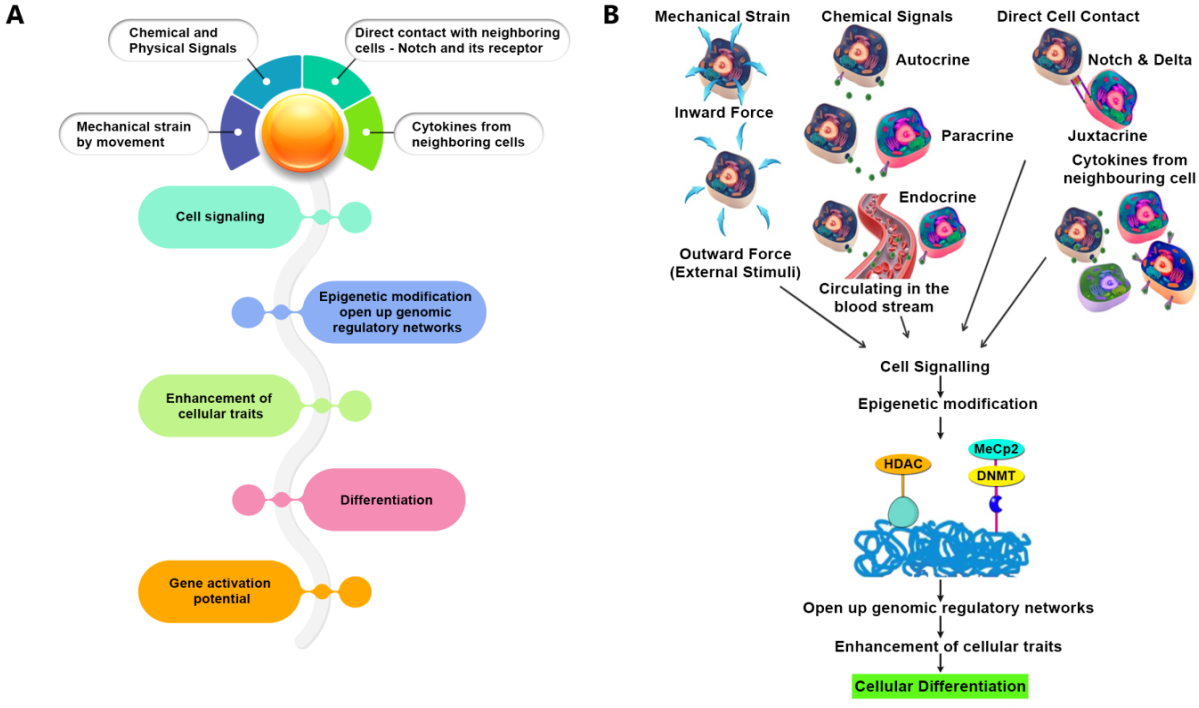

Figure 1: *(A). The cascade of differentiation leading to gene activation. As stem cells undergo differentiation, a series of molecular events unfold, ultimately resulting in the activation of specific genes responsible for the acquisition of distinct cellular identities; (B). Schematic representation illustrating various factors influencing or triggering the differentiation of stem cells. This includes environmental cues, signalling pathways, and molecular regulators that contribute to the intricate process of cellular differentiation.*

Hexa and dexamethasone compounds have also been used by Siller R *et al.* [27] for hepatic maturation. Furthermore, hepatocyte differentiation can also be accomplished by modifying the epigenetic signatures on the genomic regulatory network. The crosstalk between epigenetic modulators and transcription factors guides the eukaryotic chromatin toward hepatic lineage. Selective transcription factors may also be directly incorporated into a differentiated somatic fibroblast cell through retroviral/lentiviral vectors to achieve "direct epigenetic reprogramming" that leads to hepatocyte differentiation.

Differentiation efficiency of stem cells involves analysis of hepatic gene expression, *in vitro* hepatocyte functionality test, epigenetic analysis, global gene analysis, and *in vivo* transplantation studies. Biliary excretion indices, a membrane transporter-mediated biliary excretion, an important function of hepatocytes have been carried out by Huang P *et al.*, to prove the potential of differentiated hepatocytes for biomedical and pharmaceutical applications [32]. 2D cultures of differentiated hepatocytes are extensively used in testing drug toxicity and studying the underlying mechanism of diseases.

2D Cultures: Pros and Cons

Initial experiments carried out in the process of artificial hepatic culture relied heavily on growing cells in 2D Although this is a rapid protocol, overcomes diffusion constraints, and cost-effective, 2D culture had many limitations. For instance, cells grown in 2D cultures are flatter and more elongated than cells grown *in vivo*, affecting cell proliferation, differential gene expression, and drug uptake [33-35]. Unlike 2D monolayers, primary hepatocytes cultured in three-dimensional (3D) tissue constructs composed of multicellular aggregates maintain normal differentiated cellular function *in vitro*. The key factor which drives this difference in functionality is the 3D structure of the ECM. The ECM is a critical regulator of the cell's stemness. As well as its biomechanical properties such as geometry, stiffness, porosity, and ability to transfer mechanical signals to intracellular compartments aid in various anchorage-dependent cell behaviors such as the orientation of mitotic axes, type and rate of stem cell division, and cell migration [33,36]. These cell-to-cell and cell-to-ECM interactions play a vital role in stem cell differentiation. The genetic makeup, relevant gene activation, and mechanotransduction controlled by the

microenvironment must be synchronized to achieve specific morphogenesis [36,37]. Researchers began coating vessels with ECM materials for the cultivation of hepatocytes and other cells. These physiological 2D /2.5D culture conditions retained more physiological features of target cells than the cells differentiated in the 2D environment [38]. Thus, the hepatic ECM encloses a diverse set of cues from mature hepatocytes and non-parenchymal cells and encompasses growth-modulating molecules all of which provide the right milieu for cell differentiation. ECM is therefore a key component in the creation of a conducive microenvironment to the liver-specific commitment of stem cells. The regenerative potential of stem cells can be improved by using the appropriate biomaterial scaffolds (to provide mechanical support) for transplantation into injured regions of the liver [39]. Functional tissue formation relies on the tissue engineering triad, the cells, molecular signals, and biomaterial scaffold [40]. That being the case, recapitulation of the liver microenvironment inevitably involves stem cells, incorporation of growth factors, 3D biomaterial, transcription factors, and co-cultivation with non-parenchymal cells.

3D Cultivation Techniques

A 3D environment provides the differentiating stem cells, the volume-spatial ratio, porosity, cell-cell, and cell-ECM interactions reflecting the original niche. Several techniques are currently being studied to achieve a 3D environment mimicking an interstitial liver tissue microenvironment including; (i) Electrospinning (ii) Cell sheet engineering (iii) Encapsulation and (iv) Micropatterning which is pictorially represented in Figure 2.

Electrospinning

Using this method, natural polymers can be fabricated into scaffolds easily. Since the pore size of the scaffold can be controlled, and there is no use of high temperatures or corrosives, electrospinning is currently the preferred technique to produce nanofiber ECM scaffolds [41]. Furthermore, it avoids the loss of huge surface areas associated with ECM-coated 2.5D culture systems. These electrospun nanofibers possess high porosity and spatial interconnectivity, which is well suited for nutrient support, cell-cell, cell-ECM interactions, and other cellular responses. An electrospun composite blends synthetic polymer (as the backbone) and natural polymer (with cell recognition sites) together to achieve, a biocompatible, flexible-highly porous scaffold with tunable biodegradability and mechanical stability [42].

An optimized Poly-lactide-co-glycolide (PLGA) by wet electrospinning coupled with varying concentrations

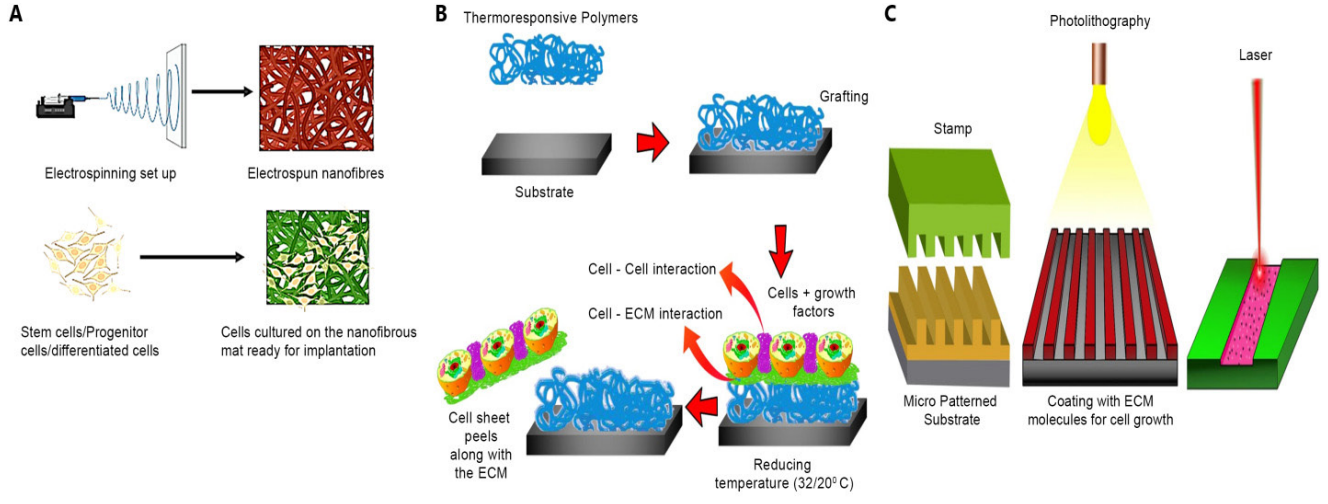

Figure 2: *(A). Electrospinning process for 3D cultivation systems. This schematic illustrates the electrospinning technique, a method utilized in the fabrication of three-dimensional cell culture systems, providing a structured and supportive environment for cell growth; (B). Pictorial representation of cell sheet engineering. This figure showcases the concept of cell sheet engineering, a technique where cells are cultured to form coherent sheets, maintaining cell-cell connections and extracellular matrix, facilitating their transfer onto biological surfaces for various applications; (C). Various methods of micropatterning or cellular patterning. This figure outlines diverse approaches for micropatterning and cellular patterning, enabling precise control over the spatial arrangement of cells in culture. These methods play a crucial role in tissue engineering and regenerative medicine.*

of collagen and fibronectin can hold primary hepatocytes with its functionality in the long term [43]. Similarly, the electrospun chitosan nanofibers along with fibronectin support the co-culture of primary hepatocytes and fibroblasts for a prolonged period [44]. Disease modeling and liver tissue engineering with such an amalgamated 3D matrix provide a viable, adaptable microenvironment for hepatocytes [45]. In an innovative study led by Upasana *et al.*, [46] an electrodeless chemical oxidative polymerization technique was employed to grow PANI (polyaniline) nanoclusters on SrO_2 (strontium peroxide) surfaces. The scaffold's mechanical properties were then improved using L-cysteine. Integration of L-cysteine-aided SrO_2-PANI nanoparticles with polyurethane (PU) polymer, using the electrospinning technique, enhanced the biological properties of the scaffold. This bioactive scaffold, promoting the elevated expression of osteoinductive markers, holds promise as a biomaterial for mimicking bone scaffolds.

Hydrogels

Hydrogels are particularly suitable for stem cell microencapsulation as they resemble the 3D aqueous-rich environment of that tissue. Encapsulation of stem cells in hydrogel provides a protective microenvironment. Hydrogels with appropriate viscous elasticity, capable of entrapping cells and allowing the interstitial flow of nutrients through diffusive transport can be used as a tissue scaffold. Besides this, stimuli-responsive (pH and temperature) hydrogels represent a promising approach as they can be injected by a minimally invasive procedure [47,48]. It is also evident that encapsulation of cells and media containing hepatic lineage-specific growth factors enhances hepatic differentiation. A recent study reported that a combination of a fiber-forming peptide component, fluorenyl methyl-oxy carbonyl-diphenylalanine, and the integrin-binding functional peptide ligand, Fmoc-arginine-glycine-aspartic acid into a nanofibrous gel at physiological pH support the primary hepatocytes with cytochrome P450 functionality [49]. The development of advanced hydrogels with explicitly designed characteristics together with nanotechnology and bioprinting enhance liver tissue engineering.

Cell Sheet Engineering

The liver comprises sheets of hepatocytes that are interconnected to form a continuous 3D tissue. As stacks of interconnected hepatocytes are required, this is not possible with the existing 3D constructs. There is also a necessity for coordination of all the soluble cytokine signals and mechanical stimuli from the liver ECM for proper liver function. To achieve a functional cell sheet with ECM, a state-of-the-art cell sheet has been developed. Such a technique potentially overcomes the drawbacks of previously available 3D constructs and the possible damages that could occur during trypsin/dispase treatment. This is highly recommended for transplantation as only the cells and ECM are implanted at the diseased site [50]. Hepatic sheet engineering involves culturing differentiated hepatocytes on a temperature-responsive polymer, poly (n-isopropyl acrylamide) which is covalently grafted onto a substrate (tissue culture plate) at 37 ℃ in a hydrophobic condition. The cell sheet is peeled off from the substrate when the temperature is reduced to 20 ℃ in a hydrophilic environment [51]. Comparably, as an advancement, Asadi M *et al.*, reported that Polyethylene Glycol (PEG)-Poly (N-isopropyl acrylamide) (PNIPAAm), a thermoresponsive polymer when combined with decellularized ECM resulted in the production of dense and intact functional hepatic cell sheets from differentiated MSCs [52].

Micropatterning

Micropatterning is one of the most widely employed techniques to attain spatial coordination of cells in an *in vitro* system. Micropatterning can be achieved by using variations in charge hydrophilicity, topology, and biomolecules separately or in conjugation with certain amino silanes [53]. Micropatterning/ cellular patterning in cell culture supports homotypic and heterotypic interactions and studies the impact of local tissue niches on biological functions. Through micropatterning, it is possible to achieve organized cell and tissue architecture [53-55]. Microfabrication technology results in numerous cellular patterns/ micropatterns (Figure 2).

As mentioned earlier, 2D culture systems exhibit various limitations, encompassing issues related to architecture, mechanotransduction, and the spatial arrangement of liver surface receptors [56]. Moreover, they struggle to incorporate crucial factors such as lower intracellular pH levels and liver polarization [34]. On the contrary, 3D culture systems have made significant strides in addressing these drawbacks by providing a more *in vivo* like environment with porous architecture and enhanced mechanical stability, these systems better emulate the complexities of liver tissue [57]. However, challenges persist, particularly in replicating intricate duct systems essential for nutrient transportation, constructing realistic blood vessels and bile ducts, and addressing metabolic zonation [58].

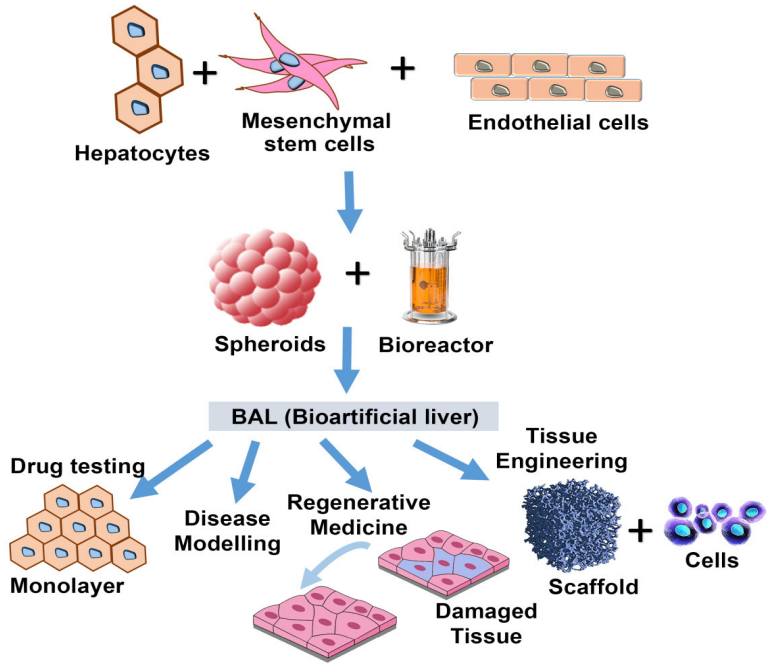

Figure 3: *Illustration depicting the developmental stages of a Bioartificial liver. The figure illustrates the stepwise progression in the creation of a Bioartificial liver, encompassing key components such as biomaterials, cell sources, and bioreactor systems, designed to mimic and support hepatic functions for potential therapeutic applications. In the second part of the figure, diverse applications of the Bioartificial liver are showcased.*

Importance of Vasculature in 2D&3D Tissue Architecture

Despite the above-mentioned advancements in the development of tissue-engineered constructs, the possibility of translation from bench to bed is still limited due to the inability to replicate mature and functional vasculature. The liver is a highly active metabolic organ, and the hepatocytes are arranged around thin-walled vascular structures called hepatic sinusoids which facilitate the easy transport of gases, solutes, and biological molecules. A major drawback with 3D culture systems and their potential for scaling up is the lack of a reliable means of transporting nutrients and oxygen throughout the system beyond what is feasible by simple diffusion [59]. Due to the absence of a perfusable vascular network, the densely populated cells in 3D-engineered tissues develop hypoxia-induced necrosis at the core. Further, a high concentration of metabolic products of the *de-novo* hepatocytes reduces cellular activity by negative feedback loops and results in cell injury. The limiting factor in determining the size of a 3D-engineered tissue is, hence, mass transport [60,61]. Vascularization in tissue engineering can be achieved by microfabrication technology, scaffold remodeling, co-culture with endothelial cells, single-depth vascular network by photolithography, and 3D planar network design. Prevascularisation techniques involve cytokines, growth factors, and/or proteins integrated into a porous scaffold which is applied to a clinical site to generate new vessels [61]. Enhancement of vascularization of a porous scaffold before transplantation has been attained by Kedem A *et al.*, [62] by sustained release of Vascular Endothelial Growth Factor (VEGF). Hepatocytes were delivered to the scaffold at the site of pre-vascularization. Ma *et al.* employed a self-polymerization process involving dopamine to enhance 3D-printed titanium (Ta) scaffolds with magnesium ions and polydopamine, resulting in the development of Mg-PDA-Ta scaffolds which promotes vascularization in bone tissue engineering. This study highlights the potential of metal-modified 3D-printed scaffolds, specifically highlighting their role in facilitating vascularization for therapeutic applications [63]. Understanding the process of angiogenesis and an improved understanding of the co-existence of various cell types, along with a dynamic control of the availability of bio factors provides an opportunity to build an optimized neovascularized tissue with a larger mass and complexity.

Liver Organoids: A Hope

During development, the liver arises from an outgrowth on the ventral wall of the foregut, that develops into the liver bud. The hepatic endoderm cells, known as hepatoblasts delaminate from this bud invading the

surrounding mesenchyme, forming hepatic stellate cells and sinusoidal endothelial cells [64]. The generation of the liver bud is accompanied by the development of the hepatic vasculature. Takebe *et al.* designed a study that demonstrated organogenetic interactions of endothelial epithelium, mesenchymal tissue aggregation, and endothelial cells [65]. The study stressed the importance of coordination of the signals in the promotion of the maturation of vascularized and functional liver buds. Human iPSCs were differentiated into hepatic endoderm (IPSC-HE) and mixed with human MSCs and Human Umbilical Vein Endothelial Cells (HUVECs). All three cell types were allowed to grow together to recapitulate the interactions during development. The team managed to create a small form of the fetal liver called "liver organoid". These organoids when ectopically transplanted into mice integrated with the host vasculature in 48 hours. Before this trial, no resultant tissues or differentiated cells integrated with the host vasculature. Moreover, the integrated buds were proven to be functional by rescuing a drug-induced lethal failure mouse model. Such iPSCs-derived liver buds/liver organoids could be used to study the pathogenetic effect of human genetic disease-causing mutations [65,66]. In another pioneering study, human liver tissue seeds were constructed using a range of cellular components co-cultured in a bio-printed scaffold. The liver seeds supported the expansion of hepatocytes when incorporated as ectopic implants after liver injury in host mice. The liver seed grafts were shown to be fully functional and considered as an alternative strategy for the scale-up of engineered organs [67].

Currently, work by several scientists has highlighted the importance of mechanotransduction in the generation of liver organoids. A recent study demonstrated a synthetic niche that was generated using Polyethylene Glycol (PEG) hydrogel for culturing liver organoids. The stiffness sensitivity of the organoids and the importance of optimization of mechanical properties of the 3D matrix were major considerations in the study. This research group successfully established a fully defined 3D culture system for mouse and human hepatic progenitors and organoids [68]. To facilitate its clinical applicability, researchers have further designed a chemically defined animal-origin-free medium for the generation and expansion of liver organoids [69]. The refined generation of liver organoids maintains the functional connection between hepatocytes and cholangiocytes. In a mouse model, Tanimizu N *et al.* generated Hepatobiliary Tubular Organoids (HBTO) containing a biliary excretion system that helps the hepatocytes to maintain their functions in the long term [70]. These concepts of organoid generation pave the way for the creation of functional *ex vivo* liver tissue. Furthermore, Liver organoids, generated through a PSC-based method, offer a reproducible platform for disease modeling. Ouchi R *et al.* demonstrated a disease model using 11 different PSC lines, including those from patients with lysosomal enzyme deficiency, these organoids recapitulate steatohepatitis pathology, allowing *in vitro* rescue with a clinically active compound via FXR agonism and providing a valuable tool for studying inflammatory processes associated with steatosis [71].

Bio Artificial Liver (BAL): A Huge Promise

BAL was developed to mimic liver-specific functions and was initially used to study tissue-specific pathogens and their role in the mechanism of disease. Matsumura *et al.* were the first to report the use of a bio-artificial liver device in a clinical setup where they used isolated hepatocytes in addition to cryopreserved rabbit liver cells in a dialyzer [72]. Although this was a breakthrough, this liver support worked only for two hours, and the cost involved in this treatment did not make this an accessible option. Recently, there has been a renewed interest in extracorporeal supportive devices, which encompass metabolically active liver cells, 3D scaffolds/ matrix, and bioreactors, performing the functions of the liver when the patient's plasma gets circulated within the device [73]. Gerlach JC described the BAL as a hybrid system that combines both functional living cells and an artificial environment [74]. A wide range of cell sources has been used in bioreactors. Predominantly used cell sources are marginal human livers, xenogeneic sources, cancer cell lines, clonally expandable stem cells, and liver progenitors' cells [75]. Similarly, biotechnologists designed efficient bioreactors which can hold both biological and artificial systems (which is pictorially represented in Figure 3).

Improvisation of bioreactors has been continued to date by biomedical scientists too. A cryogel-based cell support system developed by Lozinsky and his group has shown promise and has been considered the next generation of bioartificial livers [76]. In 2017, Selden and his group developed a BAL with alginate as the cell support system where hepatoblastoma cells were used [77]. This BAL showed a significant improvement in coagulation, reduction in vasopressor requirements, and improvement in the acute liver failure model of pigs. Yet, hepatoblastoma cells lack some of the key metabolic pathways such as those for urea synthesis. Other cells include the development of immortalized and functionally enhanced expandable liver progenitor-like cells from human primary hepatocytes called HepLPCs, capable of albumin biosynthesis and ammonia detoxification via ureagenesis [78]. These cells were loaded in an Air-Liquid Interactive Bioartificial

Liver (Ali-BAL) and were tested in a porcine model of drug overdose-induced acute liver failure, where the blood ammonia concentrations, as well as the biochemical and coagulation indices, were reduced in Ali-BAL-treated pigs. Ali-BAL treatment decreased liver damage by reducing inflammation and thereby boosting liver regeneration in the Acute Liver Failure (ALF) porcine model [79]. Overall, care must be taken in the appropriate designing of bioreactors with advanced microfluidics, the development of suitable expandable functional liver cells, and the fabrication of a suitable organotypic matrix to invent a functional bioartificial liver.

Current Clinical Practices

In this section of the review article, we emphasize and discuss the utilization of various cellular therapies for liver replacement surgeries. The major available cell-based therapeutic options of prospective clinical importance are gene therapy, cell transplantation, and bioengineered organs [80]. The major advantage of cell-based therapy is that it can be administered in a less invasive manner rather than an extensive transplantation-based procedure. Moreover, MSCs, because of their Secretome, theoretically repair the microenvironment and help in organ regeneration [81]. In clinics these cells based therapeutic options can be used for acute liver failure, liver-based metabolic disorders, regeneration after extensive hepatectomy and early fibrosis with liver stem cells or progenitor cells, bone marrow-derived- MSCs, adipose-derived MSCs, ESCs, and iPSCs. However, these approaches for both acute and chronic liver failure have not received formal healthcare approval [82].

Hepatocyte Transplantation

Hepatocyte transplantation is majorly used to treat inborn errors in metabolism (cellular gene therapy). Hepatocytes for transplantation are generally isolated from the marginal livers/ neonatal cadaveric liver by the two-step collagenase perfusion method [81,83]. Promising animal studies have shown improvements in fumarylacetoacetate hydrolase-deficient mice through serially transplanted hepatocytes [84]. The rapid immune clearance of transplanted hepatocytes by host macrophages is considered as the major drawback of this treatment [85]. The low quality of the isolated hepatocytes and the number of cells obtained are also constraints for treatment. Besides, isolated primary hepatocytes are very hard to maintain in *in vitro* conditions since they dedifferentiate within 72 hours in the absence of the appropriate milieu [86]. Furthermore, for clinically significant improvement and regeneration of the liver, approximately 10^8 cells/Kg body weight is required. This volume is currently difficult to produce *in vitro* and even more demanding to safely infuse into the hepatic vasculature. The route of infusion of these hepatocytes is via the portal circulation, a large cellular volume of infusion can potentially cause partial or total occlusion of the portal vein. This may cause portal hypertension, thrombosis, and worsening of liver dysfunction. Several safeguards against this include continuous real-time monitoring of portal venous flows and pressures and reduced rates of infusion. Innovative methods such as low-dose irradiation have also been attempted [87-89]. The exact mechanisms of interactions between the transplanted and native hepatocytes remain unknown. The availability of hepatocytes also remains a major limiting factor for this therapy. Research advancements might regulate hepatocyte transplantation therapy in the future [90,91].

Stem Cell Transplantation

With several ethical and efficacy considerations being raised for hepatocyte transplantation, stem cell transplantation is a viable alternative. Although ESCs and iPSC's can be differentiated into hepatocytes, whether they form functional native hepatocytes is not clear. Another limitation of using stem cells is their inherent capacity for extensive proliferation and the capability to form teratomas [92]. The mechanism of interaction of stem cells and native hepatocytes also remains unexplored [91]. The spatial relationship between the parenchymal cells and stem cells is not clear but plays an important role in the functional relevance of differentiated hepatocytes. Mesenchymal stem cells are not only used for trans-differentiation to hepatocytes and proliferation but can also release cytokines for the reduction of scar tissue and play a role in immunomodulation. MSCs have been shown to reduce Models for End-Stage Liver Disease (MELD) score, ascites, and overall mortality in human subjects [80]. However, unlike ESCs and iPSCs, MSCs cannot be used for long-term expansion due to the early senescence of the cells.

Bone marrow-derived stem cells, macrophages, and unsorted PBMCs are also currently being used for transplantation. Macrophages are transfused initially to reduce the scarring allowing for native regeneration. This also creates a void for transplanted cells to proliferate. CD133+ cells derived from bone marrow are transplanted via the portal vein, followed by portal venous embolization, resulting in an increased degree of regeneration, and it has potential application in treating liver cancers [93]. Scientists are now turning towards liver stem cells for the regeneration of the liver [94]. These specialized stem cells are found in the canals of Hering. Studies have also shown

extrinsic signals from circulation can replenish the function of the liver by stimulating these liver stem cells [95].

Transdifferentiated hepatocyte-like cells can either be directly injected into the site of injury or the cells must be coated on an extracorporeal liver (human or porcine origin) or 3D structures as mentioned earlier. This type of transplantation requires constant regeneration as the lifetime of hepatocytes is only 150 days; an action that needs to be performed *in vivo* or triggered externally. Another method currently followed is the injection of CD34+ cells in the hepatic artery, which requires a minimum of 2.5×10^8 cells [96,97]. Clinically hepatocyte-like cells or stem cells can be administered in extrahepatic locations like the spleen or lymph node. Takebe *et al.*, implanted 12 organoids of 300 µm each per percent weight of hepatocyte mass in the space under the kidney capsule to treat sub-acute liver failure in mice, as a pilot clinical trial [65].

In the context of regenerative research for the treatment of liver failure, adherence to a well-defined transplantation protocol is of utmost importance. The mode of administration, in this case, would involve the transplantation of regenerative cells or tissues into the affected liver. The process can be intravenous infusion of stem cells, infused into the hepatic artery, or surgical implantation of tissue-engineered constructs. Careful consideration of the administration method is crucial to ensure optimal delivery of regenerative components to the liver and to maximize the therapeutic effects while minimizing potential risks or complications [98,99]. To ensure patient safety and efficacy, strict regulations must be followed. These regulations encompass comprehensive donor screening to ensure the suitability of the cells or tissues, rigorous quality control measures to assess the viability and potency of the regenerative products, and adherence to Good Manufacturing Practices (GMP) to produce cell-based therapies [100]. The FDA regulates stem cell therapies (as a drug) through Investigational New Drug (IND) applications for clinical trials and Biological License Application (BLA) or New Drug Application (NDA) for marketing approval [101].

Ethical clearance from relevant authorities is essential to conduct clinical protocols, demonstrating the commitment to respect patients' rights, autonomy, and welfare. Ethical clearance is typically granted by an Institutional Review Board (IRB) or an Ethics Committee, whose role is to assess the scientific and ethical aspects of the proposed clinical protocols. The preclinical data obtained from small animals and primates helps clinical researchers to understand the potential benefits and risks associated with the treatment and provides crucial insights into its mechanisms of action [102]. Stem cell therapies, though promising, can carry risks such as immune reactions, tumor formation, or unintended tissue growth [103,104]. Proper risk management strategies are implemented to mitigate and address any potential adverse effects, ensuring patient safety and trial integrity. By incorporating these clinical perspectives, the research validation process gains credibility and reliability, paving the way for potential breakthroughs in liver failure treatment and fostering confidence in regenerative medicine advancements.

Progress in Liver Tissue Engineering

Today, every attempt at Liver tissue engineering aims at the development of a 3D matrix, which mimics the native liver ECM. Several such attempts were made, and we have tried to discuss a few cases in the current section. Engineered scaffolds when embedded with parenchymal and non-parenchymal liver cells mixture can be used for both testing platforms viz, xenobiotics, toxins, or as disease models and as an *in vivo* alternative for donor organs. Over the last decade, the biomedical world has seen exponential growth in the field of liver tissue engineering. In 2020, a novel cellulose nanofibril was shown to support the differentiation of liver organoids and was comparatively better than the commercially available Matrigel [105]. A liver-derived ECM hydrogel (LEMgel) synthesized by protecting the content of glycosaminoglycans, collagen, laminin, and fibronectin in the ECM of the decellularized liver that supports the physiological function of liver organoids made up of hepatocellular carcinoma cells, MSCs, and human endothelial cells [106]. Although the decellularization of organs started as early as 1948, recent advancements in the microstructural and functionality of the scaffold have been achieved by the conjugated homogenized decellularized liver [107,108]. Bioengineering of organs through decellularization and repopulation is a captivating area with significant advances and ample room for research. Even though biliary repopulation and repopulation of minority cell groups, like Kupffer and stellate cells, remain as a challenge, organoid culture may offer potential solutions [109]. In addition, numerous proteomic tools have been developed and utilized to analyze decellularized ECM that has been synthesized as hydrogels, scaffolds, and bioinks for 3D printing [110-113]. Bioprinting technology has also gained importance due to the ease of reproducing the structure of the liver. An innovative bio-ink prepared from decellularized liver ECM was used for 3D printing, which showed enhanced stem cell differentiation and maintained cellular functions of HepG2 cells. This ink was prepared without the cellular components and hence proved to be non-toxic/immunogenic [114]. By reviewing the

characteristics of bioprinting and bio fabrication strategies viz., spheroids and organoids, Sun L *et al.* demonstrated the highlights and challenges of 3D printing technology in liver tissue engineering [115]. The intrahepatic biliary network is a crucial structure that is often not formed in a matrix or hydrogel. Lewis and his colleagues demonstrated that using the decellularized porcine liver ECM with a gel matrix has resulted in the formation of intricate bile architecture [116]. As previously discussed, the importance of vascularization in liver tissue engineering, decellularized caprine liver ECM scaffold promotes angiogenesis and is a potent material for liver tissue engineering [117]. In the past few years, a great deal of work involving complex 3D structures synthesized using the latest and most advanced techniques like microfluidics, bioprinting, mimicking the hepatic plates and gaseous exchange system of the liver using CAD software, and co-culturing of hepatocytes together with endothelial and fibroblast cells for their ECM has been established [118]. A very recent novel approach, independent of 2D patterning and ECM constraints, was developed by Harrison SP *et al.*, [119] for liver-like organoid formation using a medium containing Lebovitz L-15, FBS, Tryptose phosphate broth, and small molecules. This innovative method successfully mimicked embryonic liver development, yielding organoids with a liver-like cellular repertoire, vascular structures, Kupffer cells, and functional liver features, demonstrating promising applications in drug metabolism and liver-related studies.

CONCLUSION

The current review highlights substantial progress in liver bioengineering over the last few decades. Despite advancements, the precise mechanisms that regulate liver regeneration are not fully understood. This lack of comprehensive knowledge might lead to uncontrolled cell proliferation during regeneration and raises the risk of tumor formation or limited growth and the potential for the proliferation of undesired cells within engineered scaffolds, which are of significant concerns. In parallel, the host's immunological response to the regenerated liver tissue can pose challenges and may interfere with the successful integration of regenerated tissue. Furthermore, regenerative medicine techniques that use embryonic stem cells or alteration in the genetic material of the cells could cause ethical concerns. While hurdles remain, the developments made in hepatic tissue engineering are still promising and could address the challenges associated with ESLD. Successful induced liver regeneration could reduce the dependence on liver transplants, addressing the shortage of donor organs and providing an alternative treatment option for patients. Similarly, advances in liver bioengineering may contribute to the development of personalized medicine, tailoring treatments to individual patients based on their specific needs and conditions. Advancements in tissue engineering could also explore combination therapies associated with gene therapy or nanotechnology to enhance therapeutic outcomes. Future research could lead to the creation of a 360-degree functional liver construct for transplantation, providing a more sustainable and widely available solution for ESLD.

Table 1: Differentiation conditions used to differentiate stem cells into hepatocytes/Cholangiocytes in the 2D environment.

Cells	Soluble factors/ Differentiation Conditions	Resultant cell type	Technique	Reference
Human embryonic stem cells	feeder free and EGF, IL-6, sodium taurocholate	cholangiocytes	Defined culture conditions	120
iPSCs, hepatoblasts with OP9 stromal cells	HGF, EGF and TGF-β	cholangiocytes	co-culture	121
Human iPSCs	FGF10, Activin A and retinoic acid	To obtain cholangiocytes precursor	Novel differentiation protocol	122
iPSCs	increased doses of jagged and TGF-β.	cholangiocytes	Stepwise differentiation	123
hPSCs	GSK3 inhibitor (CHIR99021), activin A, MTG, glutamine	Functional mature cholangiocytes	Matrigel differentiation	124

hESCs	dimethyl sulfoxide (DMSO), transferrin, L-ascorbic acid 2-phosphate, sesquimagnesium salt hydrate (Vc-Mg), insulin, and sodium selenite	primitive streak (PS), definitive endoderm (DE), hepatoblasts and hepatocyte-like cells (HLCs)	Chemically defined conditions	125
Extended pluripotent cells (has both embryonic and extraembryonic potential)	Activation of transcription factors f PROX1 and HNF6, which are regulated in parallel to HNF4A, FOXA2	Functional hepatocytes	Two step protocol	126
Murine and human hepatic stellate cells	ROCK inhibitors Y-33075 as well as Y-27632	Migration of hepatic stellate cells	Culture activation	127
Human pluripotent stem cells	Five small molecules FH1, FPH1, A83-01, dexamethasone and hydrocortisone, in addition to F12 basal medium and other chemicals	Functional mature hepatocytes	Three step protocol	128
PSCs, iPSCs and ESCs	BMP 4, HGF, bFGF, OSM, activin A and alternating hypoxic and normoxic conditions	Human hepatic organoids	Three step protocol on matrigel	129
Methylmalonic aciduria cblB type induced pluripotent stem cells and iPSCs	Pharmacochaperons and vitamin B12	Hepatocyte like cells	Recombinant laminins and serum free differentiation process	130
iPSCs	Activin A, BMP 4, FGF 2, HCM bullet kit for hepatocytes BMP 4,FGF 2, FGF10, retinoic acid for cholangiocytes	Electrophysiologically active hepatocytes and cholangiocytes	Three stages for hepatocytes and five stages for cholangiocytes	131
Mouse liver ductal organoids	Hnf4a, Foxa1, Prox1, and Hlf	Hepatocytes	Specific transcription factors	132

Table 2: Induced liver regeneration using 3D environments.

Cells	Technique	Materials used (Polymers)	Resultant tissue	Outcome	Reference
hESCs	Comparison of 2D and 3D	3D collagen-scaffold mimicking *in vivo* ECM 2D monolayer on collagen-coated dishes	endodermal and hepatogenic differentiation	albumin, glucose-6-phosphate was detected earlier and higher in 3D culture systems	133
Human ESCs and iPSCs	Electrospining	feeder-free Synthetic nanofibres	Hepatocytes	morphological variations during differentiation is associated with RAC1 activation	134
hMSCs	Electrospining	PLACL (Poly (l-lacticacid)-co-Polyc(e-caprolactone)/ collagen (2:1)	Hepatic-transdifferentiation	Functional hepatospheres	135
hESCs	Hydrogel	Algimatrix - a 3D culture system in association with ROCK inhibitor (Rho Kinase inhibitor)	Hepatic spheroids	3D cultures significantly improved hepatocyte differentiation and function.	136
hESCs	encapsulation of cells in alginate microbeads	HFF-CM (human fetal fibroblast-conditioned medium) along with ROCK inhibitor	definitive endoderm	Resultant cells expresses SOX17, FOXA2 and CXCR4	137
Tonsil-derived mesenchymal cells		polyethylene glycol- L-polyalanine polymer solution capable of undergoing degradation by mammalian elastases was selected to provide the modulus of 1 pKa which is like decellularized liver tissue at 37^{0}C		sol to gel transition occurs in response to temperature increase in warm-blooded animals	138

human iPSCs-derived definitive endoderm	3D micropatterning	Cultured for 14 days in the micropatterned 3D scaffold	Hepatocytes	Efficiently transferred hepatocyte-like cells displayed hepatocyte markers.	139
hESCs	3D micropatterning	blends of ECM and growth factors on a glass substrate to form 500μm spots were used	Hepatocytes	HESCs cultured on the spots for 12 days resulted in hepatocyte-like cells with early liver markers	140
HepG2 cell line	Decellularization	Decellularized Healthy and cirrhotic liver ECM	Hepatocytes	The pathological changes in the hepatic ECM microarchitecture, biochemical, and mechanical structure can favor the development of more aggressive neoplastic features.	141
HepG2 cell line	Hydrogel	fibrin (FIB) incorporated injectable alginate dialdehyde (ADA) - gelatin (G) hydrogel	Hepatocytes	Functional analysis of cultured HepG2 cells demonstrated ICG uptake, albumin synthesis, CYP-P450 expression, and ammonia clearance.	142
hiPSC-derived hepatoblasts	Microencapsulation	Alginate	Hepatocytes	They displayed a high level of albumin synthesis associated with the disappearance of α-fetoprotein (AFP) synthesis, thus demonstrating that the E-iHep-Orgs had reached a high level of maturation, similar to that of adult hepatocytes.	143
HepG2 cell line	Decellularization	decellularized caprine liver scaffold compared to native collagen scaffold (CLECM-S)	Hepatocytes	The CLECM-S compared to the collagen scaffold was pro-angiogenic and did not have any immunogenicity when implanted in a mouse model	13
HepG2 and LX2 cells, respectively), human umbilical vein endothelial cells (HUVEC)	Human liver decellularization by high shear stress oscillation-decellularization	Human acellular liver tissue cubes were synthesized from decellularized tissue	Hepatocytes	parenchymal and non-parenchymal liver cells grown in ALTCs exhibited markedly different gene expressions when compared to standard 2D cell cultures. Remarkably, HUVEC cells naturally migrated in the ECM scaffold and spontaneously repopulated the lining of decellularized vessels	144
HepG2 cell line	Hydrogel	Biodegradable and injectable in situ hydrogel formed by glycyrrhizin (GL), alginate (Alg), and calcium (Ca)	Functional Hepatocytes	Improved liver function and mRNA expression of cytochrome P450 was observed in the hydrogel	145
Stem cells and HepG2 cells	Bioprinting	Human decellularized liver ECM-based bioink was used for bioprinting	Stem cell differentiation	The bioink showed to support stem cell differentiation and maintain hepatogenic properties in the HepG2 cells.	114
Human iPSCs	Protein micropatterning	Soluble factors and ECM proteins	Maturation of iPSCs	human hepatocyte-like cells are used for disease modelling	146
Liver cells	Electrospinning	Electrospun Sodium Alginate (SA)/ Poly Vinyl Alcohol (PVA) composite coated with Ag nanoparticles	Functional hepatocytes	The 3D material shows antibacterial action in addition to the growth of nitro compounds, amides, and collagen which are the major constituents of liver tissue	147
human-induced pluripotent stem cells (hiPSCs)	Perfusion systems like bio-artificial liver	Perfusion in fresh human precision-cut liver slices (hPCLS)	Functional hepatic and biliary lineages	mRNA expression of CYP isoenzymes and transporters and the tested CYP activities, Phase II metabolism and albumin, urea, and bile acid synthesis in the hiPSC-derived cells reached values that overlap those of hPCLS, which indicates a higher degree of hepatic differentiation	148

HepG2 cell line	Electrospinning	hybrid poly-capro-lactone (PCL)-ECM scaffolds using decellularized matrix and polymer	hepatocyte growth and function	The matrix influences the gene expression profile of the HepG2s drastically and supports *in vivo* phenotype and function	149
cryopreserved PHH and non-parenchymal cells	Co-culture	3D primary human hepatocyte (PHH) spheroid	Hepatocyte function and disease modeling	hepatocyte-specific functions and under chronic exposure, the sensitivity of the hepatocytes drastically increased or disease modeling. The most interesting outcome was the chronic toxicity of fialuridine not detected in previous *in vitro* reports	150
Mouse primary hepatocytes	3D printing	Mouse primary hepatocytes were printed with alginate gel	Functional hepatocytes	Gene expression levels of Albumin, HNF-4α, and Foxa3weres were shown to increase and the 3D matrix supported long-term culture	151
Undifferentiated hepatocyte cell line (HUH7)	3D printing	3D printing of gelatin with different pore geometries - more interconnected and less interconnected	Functional hepatocytes	The hepatocyte-specific functions (albumin secretion, CYP activity, and bile transport) was shown to increase in more interconnected 3D-matrix than the less interconnected geometry and to 2D	152
Hep G2 cells	Electrospinning	Glycosylation of Polycaprolactone (PCL)/Chitosan nanofibres	Liver cells	Increased galactose with greater roughness resulted in enhanced growth and proliferation of liver cells	153
Hep G2 cells	Decellularization	Galactose-modified decellularized liver matrix	Liver tissue	Increased liver-specific metabolism and enhanced cell polarity were seen	154

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