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Bioartificial Liver Manufacturing Methodologies in Comparison to Hepatogenesis

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ABSTRACT

End-stage organ failure is a major global issue, with the liver being the second-highest transplanted organ due to lifestyle choices or other conditions. In the field of biomedical engineering, artificial organ manufacturing has been a possible alternative to organ transplants by aiming to achieve less immune rejection, more efficient production, and higher accessibility. Of the biological manufacturing methods today, two up-and-coming technologies for the liver include 3D bioprinting and decellularized organ regeneration. By analyzing general stages of 3D bioprinting and decellularization for liver development and comparing it to the cellular and genetic stages of hepatic development in the embryo, dilemmas and successes in bioartificial manufacturing can be identified. Overall, neither artificial methodology can replicate the genetic influence or non-liver based influence of hepatogenesis. 3D bioprinting is similar to hepatogenesis in cell development and construction, but contains shortcomings in vascularization, which can be addressed using the vasculogenesis and angiogenesis processes in hepatic development. Decellularization allows for cell differentiation, although it is unable to instill natural cellular distribution and gradual ECM development. This may cause blockages for clinical use in the future. Both methods have potential for bioartificial liver development and clinical application, but 3D bioprinting technology is more aligned with the stages of hepatic development, which has proven to induce fewer errors in the physiological nature of the manufactured liver. In the future, modification and research must be conducted on the specific stages of these methodologies for either to create an operational bioartificial liver for clinical use.

Introduction

Across the world, individuals suffering from deteriorating organ function or end-stage organ failure - often stemming from tumors, diseases, congenital defects/disorders, and conditions influenced by lifestyle or environment - suffer from inhibitions in physical well-being and quality of life as their bodies are unable to carry out necessary functions**.** The kidney and liver are the first and second most demanded organs for replacement worldwide: 32,586 liver transplants occurred in 2020, with thousands more on the waiting list (GODT, 2020). In the United States alone, 24,936 patients were on the waiting list for liver transplant, and 11,772 patients remained on it at the end of 2020 (SRTR, 2020).

Biomedical engineers and medical professionals have turned to the rapidly improving science of artificial organ manufacturing to address international organ regeneration necessities, especially for organs like the liver. These technologies utilize mechanics and/or biomaterials to create artificially produced organs that can be inserted into the human body. In doing so, they also aim to find more cost/time effective and/or immunologically sound solutions that re-instill organ function. Yet, a variety of issues impede the holistic success of artificial organ manufacturing, primarily in organs with anatomic and physiologic complexity. Thus far, the end products of these manufacturing technologies have been compared to naturally functional, high-demand organs

in order to see if the technologies have potential for progress in the future. However, currently popular bioartificial organ manufacturing methodologies for the liver can be compared with the stages to the natural liver development to understand possible future success and current dilemmas of such technologies. This perspective uses the means of production, not just the result, of these processes to evaluate gaps in the total construction of artificial organs.

Background on Organ Manufacturing

The process of artificial organ manufacturing utilizes a wide variety of advanced processing technologies in order to produce artificial organs. Manufacturing technologies can then employ these materials to develop artificial organs. They are constituted with recognition to biological levels of organization: molecules and polymers to functional cells, to tissues with homogeneous (same) cell composition, to organs with tissues and therefore heterogeneous (different) cell compositions. Bioartificial organs are engineered to be introduced into the patient's body, cooperating with existing internal structures to execute the necessary function of the organ in conjunction with the rest of the bodily systems by replacing the failing or malfunctioning organ physiologically and anatomically. The methods by which these organs are made are dependent upon the technologies utilized, which have varied greatly as accessibility to more biomedical technology has increased and the field of organ manufacturing continues to be explored. Such technologies must encourage the assembly of not only heterogeneous cells and growth factors, but also the extracellular matrix (ECM), which consists of various proteins and provides functional and structural support to natural tissues and organs.

Organ manufacturing as a whole has encountered several general setbacks that prevent it from becoming widespread on a permanent level. One of these dilemmas would be vascularization, or the process of developing functional, integrated blood vessels within the organ. In fact, the difficulty of vascularization is also present in the development of neural and lymphatic networks. Artificially developing operational, hierarchical networks, such as ones as small as the capillaries which are typically under 10 micrometers, is extremely difficult. These issues are present in some, but not all, organ manufacturing technologies, as will be described.

Classifying Organ Manufacturing Technologies

In order to understand the most common bioartificial organ manufacturing technologies, it is imperative that the classes of organ manufacturing technologies are clarified. There are three primary subclasses: mechanical, biomechanical, and biological/bioartificial. Mechanical technologies for organ manufacturing utilize inanimate polymers, such as plastics, and/or metals, and biomechanical technologies consist of similar materials and some living cells. Both of these subclasses provide temporary solutions to organ function failure. However, biological/bioartificial technologies are entirely centralized on using "living cells, biodegradable polymers, and/or metal elements" and utilize technology and oftentimes programs, such as computer-aided design (CAD) to assemble these biological materials; this allows for higher bodily integration and permanent restoration of organ failures or defects (Wang, 2018). Biomaterials - bioactive agents, polymers, signaling mechanisms, as well as growth factors, which primarily influence cell/tissue growth, development, and proliferation - are often used in conjunction with modern equipment. Novel research and methodologies have been executed in an attempt to successfully manufacture bioartificial organs using biological technologies with higher complexity. For the purposes of this paper, popular bioartificial/biological manufacturing technologies for the liver will be evaluated with natural liver development: their permanence and more naturally-based solutions hold more potential for complex organ implantation in the future.

Within the subclass of biological/bioartificial organ manufacturing, three further divisions of these technologies exist as well: fully automated, semi-automated, and handworked/handmade. Fully automated bi-

ological manufacturing uses entirely machine-based production to assemble biomaterials into bioartificial organs. Fully automated biological organ manufacturing technologies include additive manufacturing (AM), rapid prototyping (RP), or three-dimensional bioprinting. AM involves the development of products using extensive technology to layer (bio)materials atop one another to create a product and is often used in an industrial, as well as broader medical sense (Zadpoor & Malda, 2017). RP is a type of AM that develops scale models of CAD designs, which differs slightly from 3D bioprinting in terms of final product and methodology. However, both methods can undergo the distinct layering quality of AM, and 3D bioprinting is often considered a 'family' of RP. Semi-automated technologies include the rotational combined mold system. For the purposes of this paper and this subclass's lack of connection to current liver manufacturing, it will not be evaluated in depth. Finally, handworked/handmade organ manufacturing technologies includes decellularized organ regeneration.

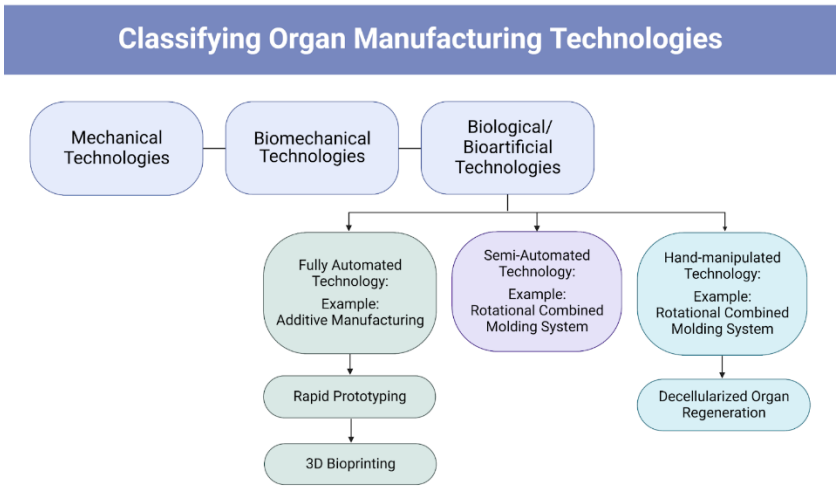

Figure 1. Classification of organ manufacturing technologies.

This paper will primarily emphasize the fully automated process of 3D bioprinting and the hand-manipulated process of decellularized organ regeneration, as notable progress has been made for liver manufacturing with these technologies.

General 3D Bioprinting Stages

3D bioprinting is considered one of the most popular and developed forms of artificial organ manufacturing thus far, especially due to its potential to create more precise microarchitecture, such as neural, lymphatic, and vascular networks. 3D bioprinting has been successfully applied, to a certain degree, to the manufacturing of tissues, organs, blood vessels, skin, bones, cartilage, the kidney, heart and liver. 3D bioprinting has not only been a focal topic in organ manufacturing due to its ability for organ implantation: its capabilities in organ manufacturing have been valuable in modeling human disease and drug discovery/toxicity as well.

There are several specific types of 3D bioprinting as they relate to certain functions, such as inkjet 3D printing, fused deposition modeling, extrusion-based liver development, stereolithography, and aerosol jet printing. Each of these methods have several specialized uses, strengths, and weaknesses, and further specific methods can be used for the total development of bioartificial human livers. In general, 3D bioprinting is beneficial due to its accuracy, capacity for dense cell-assembly, repeatability, and capacity for removed immune rejection. Its capacity for entirely controlled design proves its importance as biomedical engineers continue to standardize and specify the requirements of manufactured organs. However, it is extremely machine-dependent,

and many technologies have difficulties replicating smaller-scale, complex networks within the organ, such as functioning capillaries. Moreover, combinations of certain biomaterials, such as hydrogels and polymers, continue to be explored, leaving this prospective technology with room for growth (Wang, 2018).

3D bioprinting can be broadly described within this context as a method of pre-designing and executing the production of tissues and/or organs in an automated, layer-by-layer style, with the "most obvious feature of 3D bioprinting technology [being] the use of living cells, polymeric hydrogels, and other bioactive agents as 'bioinks' to construct bioartificial organs under the instruction of a computer-aided design" (Song et al., 2021). These "bioinks" can be assembled using a pre-designed model, ideally developing a printed organ that can precisely place a high density of living cells with regards to spatial and temporal positioning. This can not only be attributed to the fully automated nature of 3D bioprinting, which assists with general accuracy and the achievement of complex structure creation - such as vascularization - but also to polymeric hydrogels which are frequently used in 3D bioprinting to enhance biomimicry in the final organ/product, as will be described. The steps can generally be classified as follows:

Architectural Pre-Design

Predominantly, 3D bioprinting begins with a set design, often starting with CAD as a means of detailing the organ that will be constructed, then being transferred to a model. Referred to by some scientists as "architectural pre-design", this stage is vital for designing the product that will be manufactured, both physiologically and anatomically (Wang 2018). It also helps engineers and medical professionals incorporate biomimicry, loosely defined as integrating biological processes into human designing methods, thereby solving human issues (such as the international dilemma of organ failure). Biomimicry is vital in replicating natural organ function, and this paper explores its importance in the development, not just the final product, of the field of bioartificial organ manufacturing.

Preparation of Materials and Construction Tools

The next step is preparing the construction tools and materials needed to execute the manufacturing process. Preparing the tools necessary for 3D bioprinting - including the materials for the bioink - is vital to cell survival and function, as well as the functionality of the various tissues being assembled in the organ. Further biomaterials such as living cells from the patient to prevent immune rejection and stem cells that can differentiate using various growth factors are vital to 3D bioprinting preparation. Some materials must be modified beforehand, depending on requirement. For example, polymeric hydrogels, an important tool in the process, must have a plethora of traits to be operational, including but not limited to bioprocessibility, biomimetic nature, stimuli-sensitivity, and biodegradability. Polymeric hydrogels can contain and deposit several cell types at one time, and under certain conditions, can "absorb and retain a large amount of water, which is beneficial for cell growth, proliferation, differentiation, and the formation of tissues/organs" (Song et al., 2021). Polymers specifically release metabolites and supply nutrients to cells in the bioartificial organ, which further emphasizes the importance of finding adequate materials or modifying materials to be useful in preparation for the printing process. Natural and synthetic polymers in the 3D bioprinting process, a highly experimented topic in the field, are crucial to creating operational, withstanding neural, vascular, and lymphatic networks within the organ. Essentially, the bioinks are prepared for use in this stage.

Cell Assembly and Integration

The next step is usually cell assembling, in which homogeneous and then heterogeneous cell types are integrated among one another. Stem cells with growth factors and the ECM are established, with emphasis on aforementioned growth factors and the incorporation of natural/synthetic polymers to instill hierarchical network integration. The assembly and integration of these cells is vital for the functionality of the organ, with various types

of cells growing, proliferating, and operating in accordance with the organ being manufactured. Broadly stated, this stage marks the start of bioink employment by the 3D bioprinter to create the 3D organ. Oftentimes, 3D bioprinting is used to make a hydrogel scaffold, and can said technology can be used to deposit cells/bioinks in a layer-by-layer fashion that characterizes most additive manufacturing technologies.

Post Multi-Tissue Maturation

At this point in the 3D bioprinting process, the living cells have a 3D construct that can sustain an *in vivo* implant or *in vitro* culture. Structural stability is supported through the crosslinking of polymers, which immobilizes the cells and allows for the aggregation of same-celled and varied-celled tissues that comprise the organ. In doing so, the organ can achieve functional and anatomical stability as well. The following aspects of this stage are considered to be self-occurring, where stem cells differentiate depending on growth factors, and the tissues continue to become integrated with one another, inherently causing tissues to mature and become a cohesive, working structure/organ. Ideally, this stage in the 3D bioprinting process completes the organ development process through biomimicry, using the prior fully-automated stages to then somewhat naturally instill remaining physiological and structural features.

General Decellularized Organ Regeneration Stages

Decellularized organ scaffolding is another popular method of bioartificial organ manufacturing that takes a more hand-manipulated approach, compared to the fully automated proceedings of 3D bioprinting, using an outside-in approach compared to AM technology's layer-by-layer steps. Furthermore, the architectural pre-design stage of the 3D bioprinting process is not necessary in decellularized organ scaffolding: one of the primary successes of this methodology is that the pre-determined, naturally-occuring organ scaffold is provided after the decellularization protocol without a CAD design.

Decellularized organ regeneration, or decellularized organ scaffolding, is a process by which a fully formed, whole organ is removed and its viable cell contents are extracted from said organ, thereby creating an acellular organ scaffold. The scaffold can then have autologous cells (from the patient), ideally pluripotent stem cells capable of differentiation and specialization, seeded into the scaffold, and surgically implanted into the patient, using this method of recellularization to reduce immune rejection, a goal for many organ manufacturing methods. By removing cells in the original organ that may have triggered an immune response in the patient, decellularization removes these antigenic properties and hypothetically inputs cells from the patient that can be naturally accepted.

The primary goal of decellularization of the organ is to maintain the complex architecture of the organ; it not only maintains the 3D architecture of the organ and the vascularized aspects of the product, but also retains the ECM, which is invaluable to the attachment, development, and differentiation/proliferation of cells.

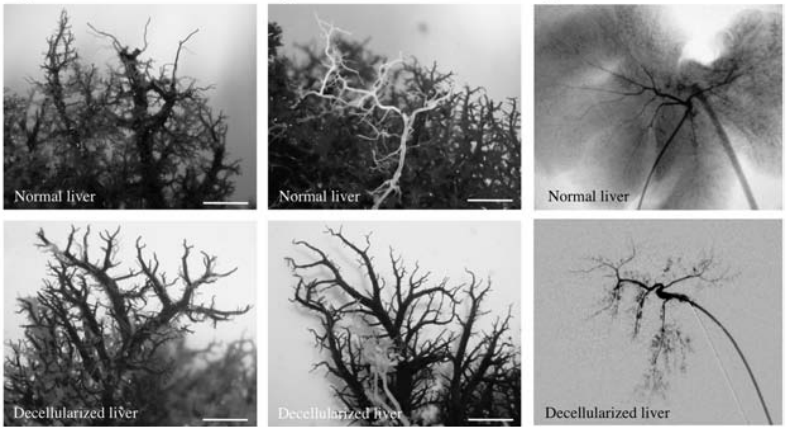

Figure 2. Radiographic imaging of normal and decellularized liver vessels and ducts. Analyzes the ability for vasculature and duct networks to withstand decellularization protocols.

Despite the unique advantages and objectives, there are several setbacks to this method. Some decellularization methods do cause impact to the ECM and 3D organ architecture, which can interfere with the cell seeding stage. Furthermore, this method still relies on other organisms for the organ itself, which can cause an increase in time and reliance on other living organisms for organ regeneration. Large amounts of cells must also be obtained and refined for recellularization purposes in order for this method to be useful in clinical settings, which can prove to be challenging and provides room for error (De Martolo & Mantovani, 2021). Some decellularization protocols cause de-endothelialization of vascular networks within an organ, and if ECM proteins come in contact with blood flowing in these vessels, coagulation could occur (Fath et al., 2021). Finally, the simple act of having multiple cell types attached to the scaffold is difficult: it requires proper cell adhesion and even spatial divide between the seeded cells for prime organ function and recellularization.

Yet, decellularized organ scaffolding has had experimental and some clinical success. Using epithelial cells and differentiated stem cells, a tracheal substitute was formed using the decellularized organ regeneration method to treat a patient with dysfunctional airways, and successfully created tubular, bioartificial organs that worked in practice, though the structure developed was not as complex as organs such as the liver or kidney (Wang, 2018). Through experimental trials, whole decellularized and cell-seeded livers were functionally implanted into rats using hiPSC (human induced pluripotent stem cells), which highlights further possibilities of using this method for humans in the future, as the setbacks continue to be addressed. Other decellularized, cell-seeded organs include the pancreas and heart.

Understanding the broad steps of organ decellularization as it currently stands can help to comprehend the shortcomings it has faced thus far, and help when analyzing this methodology from the narrowed perspective of liver manufacturing. The steps can generally be classified as follows:

Preparation and Decellularization of Organs

First, the organ taken from another organism must be decellularized to create a biological scaffold. This can either be done by surface treatment, where the organ is put into decellularizing solutions for treatment, or perfusion methods, where the decellularizing solution is put into the organ itself through the veins and arteries. Four more specific methods of decellularization include the physical, chemical, biological, and combinational method. Typically, these methods are known to use methods or agents to lyse the cells, and then rinse the scaffold to make it acellular (Crapo et al., 2011). Physically removing the living cells from the organ is easy to operate, and can remove many cells, but is often not sufficient for complete decellularization alone, and is difficult to operate in thicker tissues. The chemical method reduces the use of chemical detergents and causes a low immune response, as cells with residuality and other cell residue from the original organ are removed.

However, it does have more impact on the ECM (causing lower functionality), residual reagents used can be excreted and cause a negative response, and the growth factors of some tissues and organs are reduced as well. The biological method uses natural enzymatic methods to remove cells, but this also breaks down microstructures (which re-building can be extremely difficult), takes a long time, does not completely remove cells alone, and impacts the binding of growth factors. The combinational method uses several of these methods, and while this would completely decellularize the organ, the best combination has not been found as of now. Clearly, this stage has a wide variety of options for exploration, but has not been solidified yet. The scaffold must then be characterized as well, where atomic microscopy can be used to analyze the small-scale functionality and remnants of the ECM, as well as collagen fibers and leftover cellular debris (Hsieh et al., 2020). After one of the prior decellularization protocols are utilized, this characterization stage also places importance in checking the vascular networks: if any eluent is left, it must be removed to prevent immune rejection (Yang et al., 2022).

Stabilization of Decellularized Scaffolds

Often, the tissues of the scaffold have softened after the decellularization protocol has been completed. To increase the strength and stability of the scaffold for clinical use, the polymers can be crosslinked in the scaffold either physically, chemically, or through bio-crosslinking methods (Yang et al., 2022).

Sterilization and Preservation of Decellularized Scaffolds

Any microorganisms in the scaffold or remaining debris that can cause immune rejection in the patient must be removed prior to recellularization. In doing so, the scaffold is entirely non-toxic, which the cell-seeding process is dependent on to create an operational, safe organ for implantation. Some existing sterilization methods include "irradiation, ethylene oxide (EO), and peroxides (including peracetic acid, hydrogen peroxide, and hydrogen peroxide low-temperature plasma). Alcohol, ultraviolet light, supercritical CO_2 and antibiotics can also be used" (Yang et al., 2022). Using the accurate sterilization method depending on the organ being manufactured and the necessary functions of this organ is necessary to retain possibility for recellularization as well. Sterilization often results in impact to active, existing, acellular biomaterials in the scaffold during its execution, which is also a current struggle that scientists aim to overcome when pursuing the decellularized organ regeneration method.

The scaffold is then preserved using either cryopreservation or freeze drying to reduce possible damage when the scaffold must be used.

Recellularization (Cell-Seeding) and Implantation of Acellular Scaffold

With the scaffold being preserved, it must then be recellularized using a cell-seeding method whereby autologous cells from the patient are inserted into the scaffold. Ideally, the vascular networks of the scaffold are matched to the patient, and cells that have a lower chance of causing immune rejection are sent into the scaffold. This includes stem cells or even, in some studies, hepatocytes to evaluate the success of the scaffold itself (Hsieh et al., 2020). The scaffold can take on the large number of cells being perfused into it using its vascular structures, and cell adhesion is key to achieving a functioning organ with viable cells for the patient. *In vivo,* after the organ is surgically implanted, the vascular structures in the organ undergo anastomosis in order to become continuous with the patient's vascular network, obtaining oxygen and nutrients through blood flow**.** Through this, what is developed is a "cellular niche that contains growth factors and various signaling molecules such as proteoglycans, glycosaminoglycans, and glycoproteins" (Hsieh et al., 2020).

The biggest obstacles to this stage include heterocellular adhesion to the vascular networks, which are vital to organ functionality, and obtaining the large amount of cells needed to recellularize the organ scaffold.

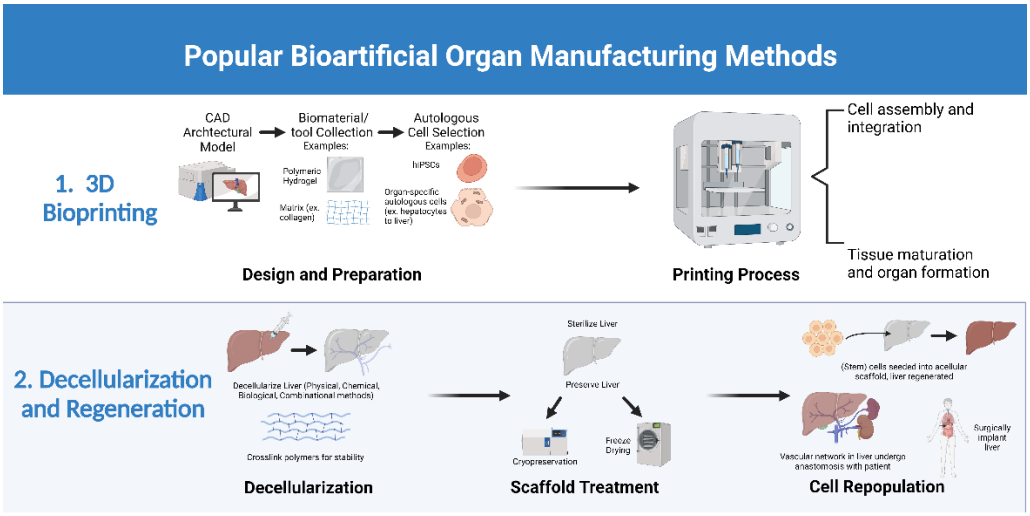

Figure 3. Comparison of 3D bioprinting and decellularization and regeneration methodologies.

Natural Liver Development

In understanding the steps of natural liver development, sometimes called hepatic development or hepatogenesis, these stages can be compared to the general and specific stages of 3D bioprinting and decellularized organ regeneration to further comprehend the similarities in the processes that have resulted in the successes and setbacks faced in bioartificial organ manufacturing. Analysis of liver anatomy and physiology and later the process during morphogenesis as the embryo continues to develop will accomplish these goals.

Liver Anatomy and Physiology

The liver is cone-shaped and approximately three pounds, with a dark reddish-brown color and located in the upper-right section of the abdominal cavity: its location often makes it prone to disease. The liver, overall, is a consistent layer of hepatocytes with infiltration by bile ducts and vascular tissue. It grossly consists of four lobes, each with microscopic, hexagonal, hepatic lobules. The lobules contain hepatocyte plates, lined with sinusoidal capillaries, that originate from a central efferent vein. The portal vein and hepatic artery of the lobules' portal triad (the third vessel being bile ducts) assist with lobule blood flow. The liver has many more networks, but for the purpose of broad understanding, it contains two major types of cells: parenchymal cells and non-parenchymal cells (parenchymal meaning relating to the (function of) tissue in an organ). The parenchymal cells of the liver are hepatocytes, which make up 70-85% of the liver's volume and carry out several vital liver functions (Song et al., 2021). Non-parenchymal cells comprise sinusoidal endothelial cells (SECs), phagocytic Kupffer cells (PFCs), and hepatic stellate cells (HSCs), among others. Other cells include pit (natural killer) cells and cholangiocytes, which are biliary epithelial cells that line bile ducts.

Of the hundreds of recognized functions of the liver, there are more commonly known functions, often relating to metabolism. This includes conversion of excess glucose into glycogen, synthesis of proteins for blood plasma, regulation of amino acids, drugs, bacteria and poisonous substances in the blood, and control of blood clotting. It holds about 13% of the body's blood at any given time, which coincides with its blood-processing functions (Hopkins, 2019).

The liver has an incredibly complex structure, specific cellular needs, and vital functions for the human body. Replicating it through bioartificial organ manufacturing methods has been difficult for this reason.

Liver Development in the Human Body

At 3-4 gestational weeks, the liver begins to develop in the ventral side of the foregut. At this point, the embryo is undergoing gastrulation, whereby the blastula, a continuous sheet of cells, forms a multi-layered structure called the gastrula. The three layers in this stage are ectoderm, meaning outermost, mesoderm, meaning middle, and endoderm, meaning innermost. These layers later on become tissues and organs through morphogenesis (development of body formation), and the endoderm is what gives rise to the liver.

Albumin is one of the markers for hepatic cell fate. The transcription factors FOXA and GATA4, located in the anterior endoderm, have the Alb gene enhancer and can bind to compacted chromatin prior to liver fate. As a result, they are referred to as “pioneer factors" indicating hepatic competence, which is the ability to respond to hepatic-inducing signals (Shin & Monga, 2013). GATA4 specifically, which is expressed in the STM by the liver bud, controls the expression of BMP4, as will be described.

The septum transversum mesenchyme (STM) disconnects from the liver bud, and cells remove themselves from the bud and enter the STM as hepatoblasts (hepatic endoderm cells that later differentiate into other hepatic cells, such as hepatocytes and biliary epithelial cells). They can intermingle with endothelial cells in the STM, which is beneficial for hepatogenesis.

The cardiogenic mesoderm, close to the liver, produces fibroblast growth factor (FGF), including FGF1, FGF2, FGF8, and FGF10, and the septum transversum creates bone morphogenetic protein (BMP), inducing expression of hepatic mRNAs during the onset of hepatogenesis. Hepatic cell fates are concentration-dependent on the hepatogenetic FGF released by the heart, so as the position liver lines more with the heart, more FGF is offered (Si-Tayeb et al., 2010). Without FGF, the pancreas would develop in the foregut instead. In the following stages, hematopoietic (blood-forming) cells enter the liver bud, causing rapid growth as it is vascularized. This process extends beyond this point and will be described in later detail, due to its vitality to liver blood filtration functions.

The hepatoblasts in the parenchyma differentiate into hepatocytes as hepatic genes are expressed in the parenchyma, maturing over time (Zorn 2008). These hepatocytes continue to mature before and after birth, developing in structure and function through several (transcription) factors. These include Oncostatin M, HGF, WNT - which has been vital to liver differentiation but continues to be explored - and glucocorticoids. Additional factors are influenced by transcription factors, including HNF4α and C/EBPα (Shin & Monga, 2013). Furthermore, hepatocytes undergo zonation, where hepatocytes conduct different functions depending on their location in the liver. The Wnt/β-catenin signaling pathway and HNF4α signaling mainly contribute to this development. Cells from the STM become fibroblasts and hepatic stellate cells, and kupffer cells come from the yolk sac.

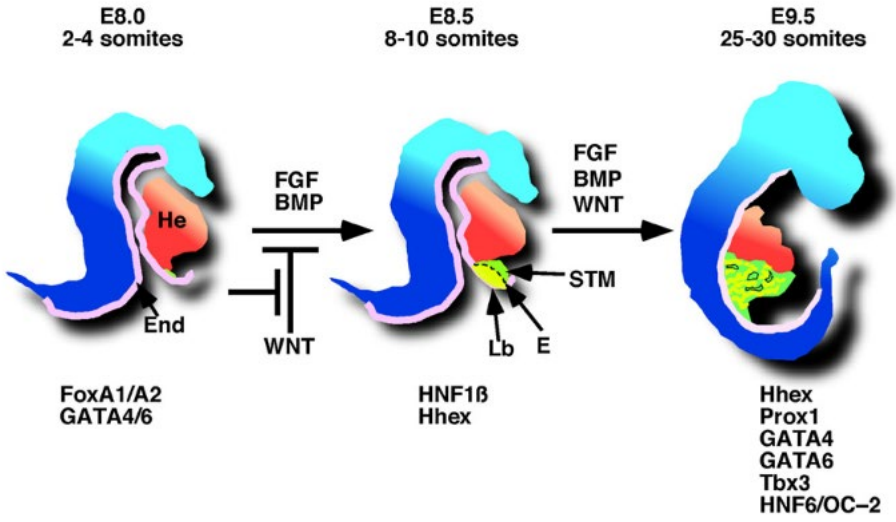

Figure 4. Genetic factors influencing hepatic development during embryogenesis.

Other hepatoblasts can differentiate into biliary epithelial cells as the biliary tract continues to mature as well. At around six weeks, hepatoblasts close to the portal mesenchyme begin biliary development from the hilum to periphery of the liver; hepatoblasts by the portal veins become biliary, intrahepatic duct endothelial cells. Genetic factors that impact extrahepatic biliary development include SOX17, PDX1, Hhex, among others. The cholangiocytes for the extrahepatic biliary ducts come from the endoderm, but hepatoblasts not only differentiate into hepatocytes, as mentioned earlier: through cell signaling SOX9, TGF-β, which initially results in hepato-biliary cells, hepatoblasts can also give rise to intrahepatic cholangiocytes (Si-Tayeb et al., 2010). The liver contributes to bile production, which makes biliary duct development vital.

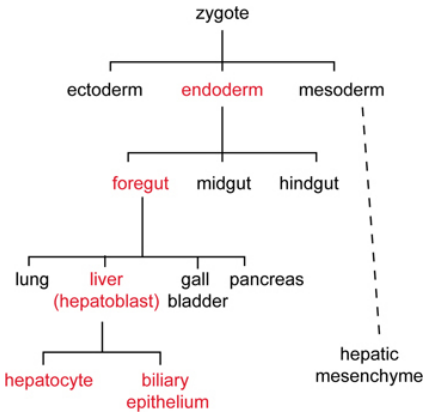

Figure 5. Hepatic commitment from the endoderm to the differentiation of hepatic cells during hepatogenesis (in red).

Finally, liver functions often incorporate blood filtration, coagulation, and other blood-related activities, not to mention that as an organ, the liver must receive nutrients and oxygen. These make vascularization extremely important for its development. The vasculature of the liver starts with vasculogenesis and angiogenesis, which develop blood vessels over time. The sinusoidal capillaries, which are highly specialized and distribute blood to the liver lobules, as well as clearing and distributing solutes, is the first to start developing in

hepatogenesis. It is also correlated with changes in the ECM as the organ matures. The fetal liver is also associated with the umbilical vein, which extends from the placenta to the heart, and the vitelline veins, which extend from the yolk sac to the heart. As the liver invades the vitelline veins, capillaries form, and left and right vitelline circulations become the left and right intrahepatic circulations as the liver matures. The umbilical veins become incorporated with the liver as the portal vein. The hepatic arteries develop afterward along the hepatic veins.

Bioartificial Organ Manufacturing Methodology Comparisons

3D Bioprinting the Liver vs. Natural Liver Development

As shown, the general steps for 3D bioprinting organs are architectural pre-design, preparation of materials and construction tools, cell assembly and integration, and post multi-tissue maturation. Several liver-specific experiments have been conducted regarding their development, and though total liver generation by 3D bioprinting has not yet been accomplished, several landmarks have occurred as of now. In 2014, Organovo developed the first 3D bioprinted human liver under the name exVivo3D™ model. Though too small for organ regeneration purposes, it is a step forward in the field of organ manufacturing, and can be used for clinical drug testing.

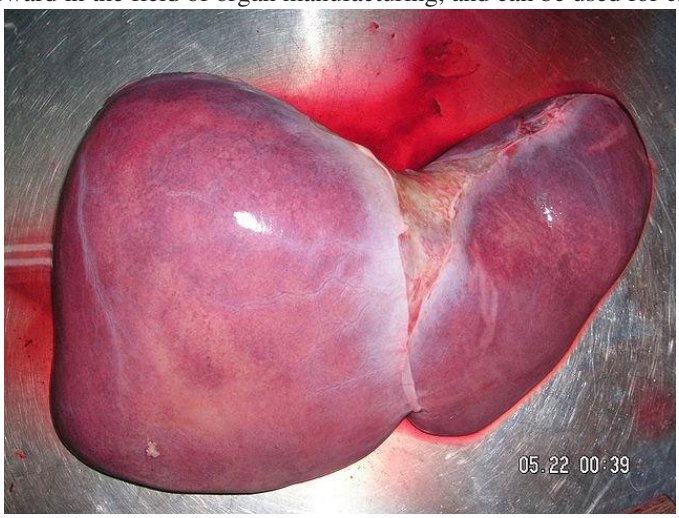

Figure 6. Organovo's exVivo3D™ mini-liver model after construction for drug testing use.

Furthermore, extensive research has been done on liver development through 3D bioprinting (though success is mainly limited to liver tissue manufacturing and restoration of livers after partial hepatectomy). Current processes for liver manufacturing include one nozzle extrusion-based 3D bioprinting and two nozzle low temperature extrusion-based 3D bioprinting for bioartificial liver manufacturing, which pushes out bioinks using the nozzles. Microstructures of organs have been developed in the liver using pressure-controlled inkjets to deposit cells and hydrogels, developing thirty layers of hepatocytes that were viable for two full months, in one study (Song et al., 2021). In another study, adipose-derived stem cells (ADSCs) which were encapsulated in hydrogels assisted with vascularization of the modeled liver, and hepatocytes were filled with hydrogels (reminiscent of the preparation of materials stage). They were then printed with growth factors to facilitate ADSC differentiation. The broader steps of these accomplishments can be compared to the stages of natural liver development (Cui et al., 2017).

First of several comparisons includes the fact that while natural hepatogenesis uses genetic and transcriptional factors to influence differentiation and maturation of cells, vessels, and ducts in the liver, 3D bioprinting utilizes an architectural pre-design stage, often using CAD programs, to influence the direction that

the printing process will take. The purpose of both the natural factors and the pre-design is to direct the development of the organ. In that sense, the function is the same, but the attainment is done through different methods. While 3D bioprinting does not consist of genetic or transcriptional factors for development of the cells, hepatogenesis uses several natural instigators, such as genes in the parenchyma and hepatic progenitors. Furthermore, in natural hepatogenesis, there is no preparation of materials or construction tools. However, this difference does not negatively impact the success of 3D bioprinting, as preparation is valuable for mechanically and biologically readying this methodology. Cell assembly and integration in 3D bioprinting is similar, in many ways functionally, to natural cell integration in hepatic development in the embryo. Cells form homocellular tissues, which then form heterocellular organs: this 3D structure is virtually always vital to the physiology of said organ. 3D bioprinting does utilize machinery to accomplish this though, while natural methods utilize other biological mechanisms. Additionally, 3D bioprinting does have a post multi-tissue maturation stage whereby tissues develop intercellularly. It's important to note that this process comes later in 3D bioprinting, which is reminiscent of cell signaling and homocellular cells coming together to form tissues, such as FGF and BMP release to genetic transcription factors such as WNT regulating the hepatic fate, which leads to tissues maturing later in the process of hepatogenesis, instead of immediately. Moreover, many molecules persist in 3D bioprinting livers, such as Albumin, one of the key markers. Another stage that remains the same is cell differentiation from stem cells or hepatoblasts, which is vital to a heterocellular, multi-functional liver. On the other hand, 3D bioprinting currently has difficulty in producing the necessary vasculature for both the self-fulfillment and the physiological properties of a working liver, whereas hepatogenesis uses vasculogenesis and angiogenesis to accomplish this important network.

The strongest similarities between the stages of 3D bioprinting and hepatic development are the ability to direct liver development, assembly of cells and tissues in a gradual manner, cell differentiation, and tissue maturation. These are vital to the organized, 3D structure of a heterocellular liver with the necessary physiological traits for implantation into a patient. As these steps continue in liver development, the fact that 3D bioprinting has incorporated them into its methodology makes it more likely to achieve a bioartificial liver with several similarities to a natural human liver. The notable difference in the methodologies include mishaps in vascularization of 3D bioprinted livers. This may be because, as previously mentioned, methods such as printing ADSCs with hydrogels do not entirely match vasculogenesis and angiogenesis, which utilize precursors to mature blood vessels to then develop mature vessels. 3D bioprinting often takes a one-time-printed approach, which uses differentiating stem cells, such as adipose derived stem cells, to print the blood vessels in one continued session. By replicating the natural vascularization of the liver, which uses older vessels to then make new vessels (a two-time-printed approach), this obstacle could be overcome. 3D bioprinting has several commonalities to hepatogenesis that open potential for a printed liver with similar anatomic and physiological capacities as a naturally developed, operational liver. However, there are differences in the methodologies that indicate room for error in the 3D bioprinting process.

Decellularized Liver Regeneration vs. Natural Liver Development

The general stages of decellularized organ regeneration are preparation/decellularization of organs, stabilization of the scaffolds, sterilizing and preserving the scaffolds, and recellularization of the organs. The liver has had significant success with decellularization, and various types of decellularization have been executed to accomplish this. The first successful case of decellularized liver regeneration used a chemical approach of detergents to decellularize the liver with surface treatment, and rat hepatocytes were seeded into the scaffold to test functionality (Damodaran & Vermette, 2018). Human stem cells have also been tested in smaller scales with rat livers, with some success in differentiation. More recently, perfusion decellularization methods have become popular, as ECM retainment has become a more achievable goal, many scientists work to focus on retaining the vasculature of the liver following decellularization. To retain scaffold structure, modern methods even include

using organ storage solutions, with another study utilizing custodiol (Dias et al., 2022). Transplants have also been a point of interest, with heterotopic transplants and orthotopic transplants being used in varying situations. Heterotopic transplants keep the chronically or acutely failing liver in the patient and insert the engineered liver as a supporting organ, while orthotopic transplants remove the liver with hepatic failure and replace it entirely with the engineered liver.

In comparing these methodologies, it is important to note that the decellularized method aims to forgo the pre-architectural design stage, or what would be the structural assignment stage in hepatogenesis, which is determined by cell signaling and genetic instruction. With that in mind, a major difference between natural hepatic development and decellularized organ regeneration is that the development of the architecture - ECM, vasculature, etc - happens in the former, not the latter.

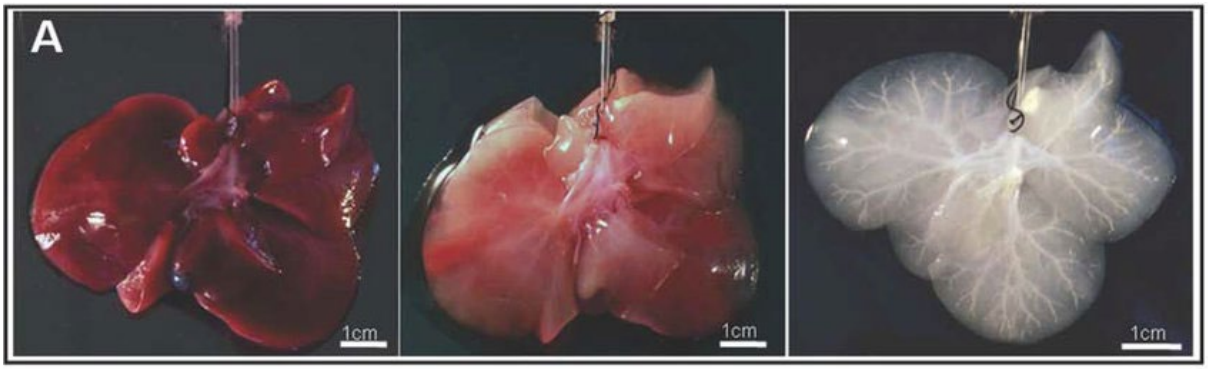

Figure 7. Maintenance of 3D architecture and vasculature of liver tissue following decellularization protocol.

Decellularization methods may harm the soluble aspects of the ECM, and though they retain many aspects of the vascular systems, it is not foolproof yet. The ECM influences liver function, as it does with countless other cell types, and for that reason, this methodological difference can cause a not-entirely functional liver scaffold, which is currently being addressed through experimentation and research. As mentioned though, decellularized organ regeneration is extremely useful in its ability to mostly maintain the 3D architecture of the donated organ, which is difficult with several other mechanical methods. Stabilizing the scaffolds by crosslinking polymers, sterilizing them, and preserving them are not in hepatogenesis, but these stages of decellularized organ regeneration are beneficial to the patient for immunological reasons and anatomical reasons. Decellularized organ regeneration also may have struggles with zonation in the liver. In natural hepatogenesis, concentration of cells depending on location is managed by genetic instruction, cell signaling, etc. However, while recellularizing the acellular scaffold through veins and arteries, it is very difficult to regulate the concentration of cells and their placement in the organ, which can lead to errors in the cell seeding process. Decellularization does allow for cell differentiation and maturation after recellularization, though, which is a vital step in hepatogenesis to ensure the liver is physiologically sound, as pluripotent stem cells are ideally seeded into the decellularized scaffold.

Decellularization and hepatogenesis share cellular development properties, and allow for cellular differentiation by using patient autologous stem cells. They are also very similar in that vasculature and ECM architecture and prominent in their end products. Yet, methodologically, there are several differences, including lack of development of architecture, which can lead to deterioration of the ECM and vasculature, and problems with distribution of cells. These issues can lead to lack of functionality: ECM deterioration damages cell function, lack of vasculature means that the liver may have trouble carrying out blood filtering functions, and improper cell distribution can mean issues with zonation and overall cell interactions. Decellularization overcomes many design issues that other organ manufacturing technologies have, but its lack of coordination with natural

hepatogenesis also indicates several issues that could arise with the process as well. Overall, this technology has high potential, but its methodologies open several rooms for error.

Conclusion

In brief, by analyzing the methodologies of popular liver manufacturing techniques and comparing them to natural hepatic development, the successes and shortcomings of such techniques can be evaluated in order to examine future treatments for liver failure. The compared factors included gradual development of 3D architecture, cell differentiation and distribution, tissue maturation, vascularization, among others. The general and specific classifications/methods of 3D bioprinting and decellularized organ regeneration were summarized and qualified in comparison to the broad development of the liver through hepatogenesis in the liver. Following this review, it was clear that 3D bioprinting consisted of similarities in terms of determination and assembly of cells in the liver to hepatogenesis, while differences occurred in vascularization. However, decellularized organ regeneration had discrepancies with architecture development and distribution of cells; it did, however, work to maintain the ECM and vasculature of the liver, and allowed for stem cell differentiation and interaction. All things considered, both organ manufacturing methodologies have potential for use in the field of liver manufacturing, but 3D bioprinting, in having more methodology similarities to hepatic development, is more solid in its progress thus far. When evaluating how to incorporate it for clinical use, overcoming the remaining manufacturing difficulties may require a comparison to natural hepatic development in order to modify existing manufacturing techniques. In discussing modern methods, many manufacturing techniques analyze the final product of hepatogenesis and develop a method that is not founded on the principles of natural organ development. This paper emphasizes the need for the fields of biomedical engineering and organ manufacturing to become increasingly intertwined with the stages of hepatic development in order to examine how to develop an entirely functional liver.

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References

Crapo, P. M., Gilbert, T. W., & Badylak, S. F. (2011). An overview of tissue and whole organ decellularization processes. *Biomaterials*, *32*(12), 3233–3243. https://doi.org/10.1016/j.biomaterials.2011.01.057

Cui, H., Nowicki, M., Fisher, J. P., & Zhang, L. G. (2016). 3D bioprinting for organ regeneration. *Advanced Healthcare Materials*, *6*(1). https://doi.org/10.1002/adhm.201601118

De Bartolo, L., & Mantovani, D. (2021). Bioartificial Organs: Ongoing Research and future trends. *Cells Tissues Organs*, 125–127. https://doi.org/10.1159/000518251

Dias, M. L., Paranhos, B. A., & Goldenberg, R. C. (2022). Liver scaffolds obtained by decellularization: A transplant perspective in liver bioengineering. *Journal of Tissue Engineering*, *13*, 204173142211053. https://doi.org/10.1177/20417314221105305

Evans, D. W. (2011, December). *A nanoindentation device and the scale-dependent mechanical properties ...* Retrieved August 8, 2022, from https://wakespace.lib.wfu.edu/bitstream/handle/10339/36435/Evans_wfu_0248M_10213.pdf

Fathi, I., Imura, T., Inagaki, A., Nakamura, Y., Nabawi, A., & Goto, M. (2021). Decellularized whole-organ pre-vascularization: A novel approach for organogenesis. *Frontiers in Bioengineering and Biotechnology*, *9*. https://doi.org/10.3389/fbioe.2021.756755

Guruswamy Damodaran, R., & Vermette, P. (2018). Tissue and organ decellularization in Regenerative Medicine. *Biotechnology Progress*, *34*(6), 1494–1505. https://doi.org/10.1002/btpr.2699

Hsieh, D.-J., Srinivasan, P., Yen, K.-C., Yeh, Y.-C., Chen, Y.-J., Wang, H.-C., & Tarng, Y.-W. (2021). Protocols for the preparation and characterization of decellularized tissue and organ scaffolds for tissue engineering. *BioTechniques*, *70*(2), 107–115. https://doi.org/10.2144/btn-2020-0141

Liver: Anatomy and functions. Johns Hopkins Medicine. (2019, November 19). Retrieved August 8, 2022, from https://www.hopkinsmedicine.org/health/conditions-and-diseases/liver-anatomy-and-functions

Scutti, S. (2013, November 11). *First 3D printed liver: Will transplantable human organs eventually be bioprinted?* Medical Daily. Retrieved August 8, 2022, from https://www.medicaldaily.com/first-3d-printed-liver-mini-mighty-could-transplantable-human-organs-eventually-be-bioprinted-262565

Shin, D., & Monga, S. P. (2013). Cellular and molecular basis of liver development. *Comprehensive Physiology*, *3*(2), 799–815. https://doi.org/10.1002/cphy.c120022

Si-Tayeb, K., Lemaigre, F. P., & Duncan, S. A. (2010). Organogenesis and development of the liver. *Developmental Cell*, *18*(2), 175–189. https://doi.org/10.1016/j.devcel.2010.01.011

Song, D., Xu, Y., Liu, S., Wen, L., & Wang, X. (2021). Progress of 3D bioprinting in organ manufacturing. *Polymers*, *13*(18). https://doi.org/10.3390/polym13183178

U.S. Department of Health and Human Services. (2020). *OPTN/SRTR 2020 Annual data report: Liver* . Health Resources and Services Administration. Retrieved August 8, 2022, from https://srtr.transplant.hrsa.gov/annual_reports/2020/Liver.aspx

Wang, X. (2018). Bioartificial Organ Manufacturing Technologies. *Cell Transplantation*, *28*(1), 5–17. doi: 10.1177/0963689718809918

World Health Organization. (2020, December). *2020 International Activities Report*. Global Observatory on Donation and Transplantation. Retrieved August 8, 2022, from http://www.transplant-observatory.org/2020-international-activities-report/

Yagi, H., Fukumitsu, K., Fukuda, K., Kitago, M., Shinoda, M., Obara, H., Itano, O., Kawachi, S., Tanabe, M., Coudriet, G. M., Piganelli, J. D., Gilbert, T. W., Soto-Gutierrez, A., & Kitagawa, Y. (2013). Human-scale whole-organ bioengineering for Liver Transplantation: A regenerative medicine approach. Cell Transplantation, 22(2), 231–242. https://doi.org/10.3727/096368912x654939

Yang, J., Xu, Y., Dang, H., & Han, H. (2022). Preparation and research progress of tissue-organ decellularized scaffolds. *Chinese Journal of Biotechnology*, *38*(6), 2169–2186. https://doi.org/10.13345/j.cjb.210772

Zadpoor, A. A., & Malda, J. (2017). Additive manufacturing of biomaterials, tissues, and organs. *Annals of Biomedical Engineering*, *45*(1), 1–11. doi: 10.1007/s10439-016-1719-y

Zorn, A. M. (2008). Liver development. *StemBook*. https://doi.org/10.3824/stembook.1.25.1