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Stem Cells for HUMAN Hepatic Tissue Engineering

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1. Introduction

1.1 Clinical implications

End-stage liver disease is a life-threatening condition for which the only effective medical treatment available to date is orthotropic liver transplantation. Other approaches are needed because of the severe shortage of donors. These alternatives include cell transplantation and extracorporeal bioartificial livers. Since adult hepatocytes do not readily proliferate in culture, and healthy human livers are used to meet transplantation needs and are therefore not available for other purposes, a major challenge for these cell-based therapies is to identify a reliable hepatocyte source[1]. In the case of extracorporeal devices, many have suggested the use of animal sources (e.g. rat and pig), where immunoisolation may be possible. This is not likely to be a viable option for cell implantable modalities since these implants must be vascularized to function properly, and as a result would be exposed to xenogeneic immune rejection[2]. An increasingly plausible approach is the use of hepatocytes derived from embryonic stem cells (ESCs), as recent studies – reviewed in greater detail below – show that it is possible to differentiate ESCs into hepatocyte-like cells with high yields. Furthermore, recent developments with induced pluripotent stem cells (iPSCs) suggest that many of the procedures used to differentiate ESCs could be used on iPSCs, thus making it possible to derive patient-specific syngeneic hepatocytes. Besides therapeutic applications, hepatocytes derived from human ESCs can be used for a variety of other applications, such as toxicity drug screening, where the use of human cells is much preferred compared to animal cells that often vary in sensitivity and metabolism of xenobiotics and drugs.

1.2 Industrial implications

One of the fundamental challenges facing the development of new chemical entities within the pharmaceutical industry is the extrapolation of key in vivo parameters from in vitro cell culture assays and animal studies. Development of microscale devices and screening assays incorporating primary human cells can potentially provide better, faster and more efficient prediction of in vivo toxicity and clinical drug performance[3]. With this goal in mind, large strides have been made in the area of microfluidics to provide in vitro surrogates that are

designed to mimic the physiological architecture and dynamics. Current embodiments of this synergy cover various microelectromechanical (MEMs) devices that contain hepatocytes, for use in drug metabolism screening and toxicology assessment.

1.3 Liver function and structure

The liver, a key metabolic and detoxification center, contains parenchymal cells called hepatocytes (70%) and various nonparenchymal cell types such as sinusoidal endothelial cells, stellate (fat-storing, Ito) cells, Kupffer cells, and cholangiocytes (bile duct cells). Hepatocytes are responsible for most liver-specific functions, including albumin synthesis, detoxification of ammonia into urea and glutamine, bile and cholesterol production [4]. The liver is also critical for maintaining circulating glucose levels via gluconeogenesis during fasting. A large array of enzymes is responsible for the detoxification of organic compounds, either endogenous (such as many hormones) or exogenous (drugs and toxins) via two sequential mechanisms described as Phase I and Phase II biotransformations. There is an extensive body of literature that has shown that controlling environmental parameters, in other words the culture conditions, which consist of the type of substrate used, spatial orientation of the cultured cells, addition of growth factors, and the combinatorial effects of these parameters, is critically important to induce and maintain a high level of hepatocellular viability and function[5]. The functional and structural complexity of the liver organ has been very difficult to reproduce ex vivo in hepatocyte culture systems. Some of these challenges have been partially met using novel cell culture approaches as well as microfabrication techniques that can emulate the size scales of the liver sinusoid. While all of these studies describe various techniques to boost in vitro hepatocyte function, they do not resolve the limited access to primary hepatocytes, which do not proliferate to any significant degree outside of the liver[1]. This is crucial because despite modifications in the culture environment, large numbers of mature hepatocytes are needed, and yet, are not available, for clinical applicability. Herein, we focus primarily on the challenge of securing a sufficient supply of high functioning hepatocytes for clinical and industrial applications using a stem cell differentiation platform.

1.4 Adult stem cells

To address cell source issues more effectively, research into alternate hepatocyte precursor populations has been conducted. Unlike differentiated cells, hepatoblasts are not only capable of expressing differentiated function, but are also able to self-renew. A few hepatoblasts have been identified that have the capacity to differentiate into mature hepatocytes and include bipotential precursors for hepatocytes and biliary cells, and hematopoietic stem cells [6].

In scenarios following severe hepatic injury, liver regeneration is attributed to a potential stem cell compartment located within the smallest branches of the intrahepatic biliary tree, which gives rise to the bipotential cells known as oval cells [7]; [8]. Oval cells are characterized as small cells with a high nucleus-to-cytoplasm ratio, oval shaped nucleus, and the ability to express markers of both fetal hepatocytes and biliary cells [9]; [10]. Oval cells have been shown to require growth factors such as transforming growth factor alpha (TGFα), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) for progression through the cell cycle as well and subsequent differentiation toward mature hepatocytes [11]. Despite the large number of observations describing liver growth

processes driven by oval cell proliferation and differentiation into hepatocytes, oval cells are difficult to isolate and the molecular mechanisms behind these processes must still be elucidated.

Hematopoietic stem cells (HSCs) have also been induced to differentiate along hepatocyte specific pathways. For example, one experimental system utilized HSC transplantation to alleviate liver disease in fumarylacetoacetate hydrolase (FAH) deficient mice [12]. FAH deficiency leads to liver dysfunction and eventual lethality. Following HSC transplantation, liver function was reconstituted. However, it is unclear whether the HSCs or HSC progeny that repopulated the liver. In addition, the mechanism that induces differentiation toward mature hepatocytes is unclear.

Despite the fact that hepatoblasts exhibit the potential to provide a renewable hepatocyte cell source, these cells are hard to isolate and exist in very low numbers [13]. In addition, the full efficacy of utilizing these precursor cells is questionable, since the long-term functional stability of hepatocytes obtained from these systems has yet to be assessed.

2. Embryonic stem cells and induced pluripotent stem cells

There are multiple stem cell starting paths for hepatocytes: 1. ESCs, 2. iPSCs, 3. Endoderm precursors and 4. Hepatic stem cells. However, due to their robust nature and large body of literature, we will focus on the first two. Furthermore these two cell types are readily abundant and have a higher proliferative capacity, thereby providing a strong potential starting point for the aforementioned applications.

2.0.1 Embryonic stem cells (ES cells)

Embryonic stem cells, derived from the inner cell mass of the blastocyst [14], have been proposed as another potential source for the generation of mature hepatocytes. ES cells are pluripotent and can be induced to differentiate into any cell type. When cultured in the presence of an anti-differentiation agent such as leukemia inhibitory factor (LIF) and with or without a feeder layer, these cells can proliferate while maintaining pluripotency [15]. Upon removal of the anti-differentiation agent, ES cells begin to spontaneously differentiate.

2.0.2 Induced pluripotent stem cells (iPS cells)

A new area of research has developed in recent years, majorly in part due to the legislative restrictions certain countries have placed on ES cell research. One area that has shown strong advancement is the area of induced pluripotent stem cells (iPS cells). iPS cells are the result of reprogramming somatic cells to a pluripotent state which resemble ES cells with respect to morphology, proliferation (self- renewal), surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, epigenomics and telomerase activity [16, 17]. Human iPS cells' autologous nature offers several advantages over human ES cells in regards to potential patient specific therapeutics, the study of disease state, study of developmental processes, drug discovery as well as drug toxicity on differentiated hepatocytes while avoiding the ethical issues associated with isolation and the usage of human ES cells as illustrated in Figure 1.

Yamanaka coined the term induced pluripotent stem cells in 2006 while inducing a pluripotent state in mouse somatic cells by direct reprogramming [16]. The same year, his lab demonstrated the generation of induced pluripotent stem cells from adult human

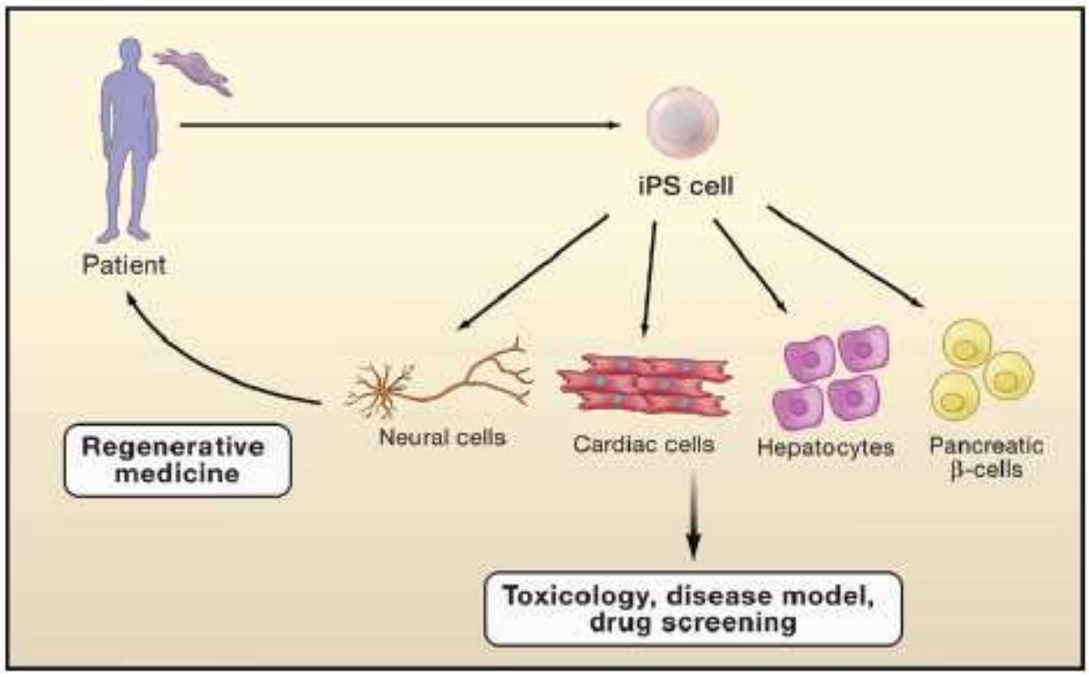


Fig. 1. **Applications of IPS Cell Technology**
Patient-derived iPS cells can produce various somatic cells with the same genetic information as the patient. These cells could be used to construct disease models and to screen for effective and safe drugs, as well as to treat patients through cell transplantation therapy. (Figure taken from [20], figure 1)

fibroblasts by retroviral transfection of four factors: Oct3/4, Sox2, c-Myc and Klf4 [16, 18]. Since then, others have generated human iPS cells using different combination of factors while preserving Oct3/4 and Sox2 as the core factors needed for pluripotency reprogramming while Oct3/4 is the most important[19, 20]. The main risk associated with the use of human iPS derived cells for transplantation is directly influenced by the iPS generation methods which may involve risk of DNA modification (gene deletion, viral gene incorporating into the human genome and gene expression alterations) which may lead to insertional mutations that will affect iPS function, differentiation potential and tumorigenesis [20, 21]. The human iPS cells generated by the Yamanaka group, for example, had more than 20 retroviral integration sites in total which may increase tumor formation [16, 18]. That risk has accelerated the search for new methods for generating clinically safer iPS cells such as the use of non-integrating episomal vectors or the use of molecules that promote or enhance reprogramming of somatic cells to iPS cells to improve reprogramming efficiency and reduce genomic alterations due to viral integration [21, 22].

2.0.3 Induced pluripotent stem cells are similar, but not identical to embryonic stem cells

Although iPS cells share similar features with ES cells as mentioned above, they are not identical. The reprogramming process does not involve genetic transformation but rather an epigenomic one. Lister et al. have shown that iPSCs are not identical to ESCs with respect to epigenomic profile by profiling whole-genome DNA methylation at single-base resolution

in five human iPSC lines, along with methylomes of ES cells, somatic cells, and differentiated iPSCs and ES cells [17]. In addition, iPSCs maintain some epigenomic features which resemble the somatic cell they have been generated from [17]. Cell memory based on epigenomics may suggest that, for example, iPSCs which were generated from primary hepatocytes as a somatic cell source will yield more or better functioning iPSC derived hepatocytes compared to those generated from iPSCs which were generated from fibroblasts as a somatic cell source. These discrepancies between human ES and iPS cells led to the question of what is the difference between the two stem cell types in regards to pluripotency and differentiation potential? And what is the best method to assess it? The ultimate test for pluripotency in the mouse system is the generation of a chimeric mouse which was successfully generated by blastocyst microinjection of mouse ES and iPS cells. Obviously, this test is not applicable to human ES and iPS cells and, therefore, other methods are being used to determine the pluripotency of the cells such as teratoma formation. As in the case of human ES cells, human iPS cells have the potential to differentiate to any of the three germ layers and form teratomas when transplanted subcutaneously into a severe combined immunodefficient (SCID) mouse [16, 18]. Unfortunately, teratoma formation does not guarantee full reprogramming as many mouse ES cell-like cell lines form teratomas but fail to produce germline chimeras [20]. The ultimate method to assess the differentiation potential of human ES and iPS cells to mature and functional hepatocytes is to compare the two with respect to mature hepatocytes gene and protein expression profiles, as well as metabolic activity, drug clearance, glycogen storage, urea and albumin synthesis and secretion while using the same differentiation method.

In addition to the functionality of the differentiated hepatocytes, the efficiency of the process is of high importance due to the potential of human ESCs and iPSCs to differentiate spontaneously into cells from the three germ layers. Complete differentiation of stem cells in vitro is especially important when used for cell based therapies. Heterogeneous populations at the end of the differentiation process composed of differentiated cells as well as stem cells may lead to teratoma formation at the site of transplantation as well at other locations to which the cells migrated.

2.1 Traditional embryonic stem cell and induced pluripotent stem cell differentiation paradigms

Many paradigms currently exist to specifically direct the differentiation of embryonic stem cells toward a hepatocyte lineage in vitro, while utilizing the knowledge of embryological pathways occurring in vivo during normal liver development. This process involves numerous stages and is influenced by cytokines as well as cell-matrix interactions in a temporal and spatial manner. When developing new paradigms for direct differentiation of human ES and iPS cells into mature and functional hepatocytes in vitro with high efficiency, one must refer to the developmental process of the liver during embryonic development.

2.1.1 Hepatocyte differentiation during embryogenesis

The main stages are illustrated in Figure 2A. In the first phase, ES cells differentiate to endodermal cells. ES and iPS cells are pluripotent and therefore can give rise to any of the three germ layers: ectoderm, mesoderm and endoderm where the latter, more specifically the anterior- ventral definitive endoderm, give rise to the cells of the liver. The expression of

Wnt signaling inhibitors in the anterior endoderm represses the Wnt/β catenin pathway and was shown to be required for liver specification in the endoderm [23]. Signaling by the transforming growth factor beta (TGF*β)* growth factor Nodal at relatively high concentrations initiates endoderm formation. Nodal signaling stimulates the expression of a core group of endoderm transcription factors including the HMG domain DNA-binding factor Sox17 and the fork head domain proteins Foxa1–3 (HNF3*α/β/γ*) which in turn regulate a cascade of genes committing cells to the endoderm lineage [24]. FoxA2 and GATA4 serve as transcription factors for the alb1 gene which encodes for serum albumin and appear early in the pre-liver hepatic domain of ventral foregut endoderm and later in liver [25, 26].

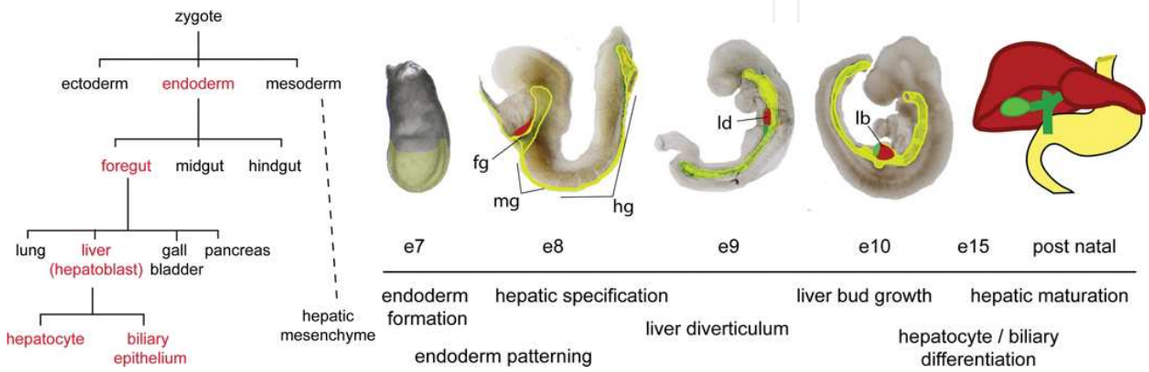


Fig. 2. **Embryonic development of the liver.**
(A) Development of the liver is illustrated from the standpoint of cellular differentiation (red) from uncommitted endoderm to functional adult hepatocytes and biliary epithelium. (B) The schematic shows mouse embryos at different stages of development with the endoderm tissue highlighted in yellow, the liver in red, and the gall bladder in green. The major developmental events are listed below. The endoderm germ layer is formed during gastrulation (e6.5-e7.5). Throughout gastrulation and early somite stages of development (e7-e8.5) the endoderm is patterned along the A-P axis into foregut (fg) midgut (mg) and hindgut (hg) progenitor domains. Morphogenesis forms foregut and hindgut pockets as the endodermal cup is transformed into a gut tube. By e8.5 hepatic fate specified in a portion of the ventral foregut endoderm adjacent to the heart. As the embryo grows the endoderm forms a gut tube and the liver domain moves to the midgut. The liver diverticulum (ld) forms by e9 and expands into an obvious liver bud (lb) by e10. The liver grows, and by e15 hepatoblasts are differentiating into hepatocyte and biliary cells. Final maturation of the liver is gradual and continues into the postnatal period.
(Figure taken from[74], figures 1 and 2)

In the second phase, we see the differentiation from definitive to hepatic endoderm. The onset of liver development is characterized by the commitment of midgut endoderm to become liver through interactions with cardiac mesoderm which secrete fibroblast growth factors (FGFs) [27]. FGFs signaling in the foregut endoderm activated the MAPK pathway is necessary for initiation and stabilization of hepatic differentiation [28]. Bone morphogenetic proteins (BMPs) (BMP2, BMP4, BMP5, and BMP7) signaling from the septum transversum mesenchyme also contributes to hepatic gene induction in the endoderm [29-32]. Wnt

signaling along this stage is suppressed but is required in the following stage where the hepatic endoderm outgrows into the liver bud[23]. In addition, HNF3β and activin A signaling are involved in the process of the specification from endodermal stem cells toward the hepatic epithelial lineages as indicated in Figures 2 and 3 [31, 32]. Under the influence of these signals, endodermal cells (the liver bud) migrate from the ventral foregut into the extracellular matrix (ECM) rich septum traversum, forming the primordial hepatoblasts. This migration is accompanied by major remodeling of the extracellular matrix surrounding the hepatic cells. Some investigators have tried to induce this process in vitro by differentiating ES and iPS cells using various types and configurations of ECM [33].

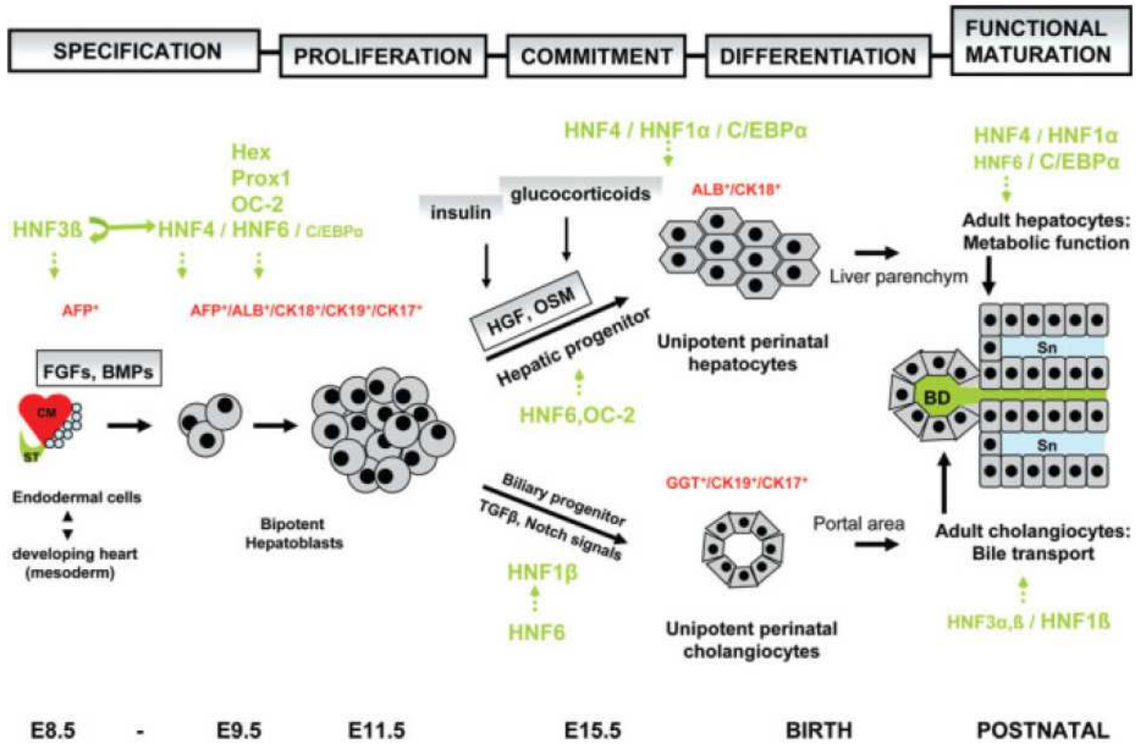


Fig. 3. **Schematic presentation of fetal liver development.** The establishment of a fully functional liver architecture is not accomplished before postnatal stages and follows upon a sequential array of tightly regulated intra- and extracellular signaling pathways, including liver-enriched transcription factors (LETFs) and growth factors, cytokines, glucocorticoids and hormones, respectively. To distinguish the level of expression and/or regulating role among diverse LETFs, different letter sizes are used. Abbreviations: ALB, albumin; AFP, alpha-fetoprotein, BMP, bone morphogenic proteins; C/EBP, CCAAT enhancer binding protein; CK, cytokeratin; CM, cardiogenic mesoderm; E, embryonic day in rodent liver development; FGF, fibroblast growth factors; GGT, c-glutamyltransferase; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; OC-2, Onecut transcription factor; ST, septum transversum; TGF, transforming growth factor. (Figure taken from[31], figure 2)

In the third phase, the liver primordium is induced to invade the septum transversum, giving rise to fetal hepatocytes (primordial hepatoblasts) which are bi-potent and may

proliferate of later differentiate into hepatocytes and cholangiocytes (biliary cells). Markers of hepatoblasts inherent in this phase are GATA4, HNF4alpha, HNF6, hepatic alpha-fetoprotein [AFP], albumin [ALB], and biliary cytokeratin (CK 17/CK19) [29, 30, 32].
Hematopoietic stem cells (HSCs) colonize the liver bud, thereby emitting a growth signal for the liver. Hematopoietic tissue and hepatoplasts subsequently release additional growth factors to further develop both tissues. Hepatoblasts continue to proliferate and start expressing placental alkaline phosphatase, intermediate filament proteins (cytokeratins CK14, CK8, and CK18), c-glutamyltransferase, and later also alpha1-antitrypsin, glutathione S-transferase P, C/EBPα, lactate dehydrogenase, and muscle pyruvate kinase [29-32].
At this stage three cell populations exist: (a) hepatocyte-committed cells that exclusively express hepatocyte markers, such as AFP and ALB, (b) cholangiocyte-committed progenitor cells, expressing biliary cell markers such as CK19, and (c) a bipotential hepatoblast population, expressing both hepatic and biliary markers. Bipotential hepatoblasts then proceed through a series of maturation steps which entail proliferation, cellular growth, and functional maturation. These final in vivo steps are induced by various extracellular signals such as: (1) dexamethasone, which induces albumin production and downregulates alpha-fetal protein production; (2) transforming growth factor beta, which inhibits hepatocyte proliferation and increases albumin production; (3) oncostatin M, mostly produced by HSCs, which induces tight cell-cell contacts, necessary for maximum differentiated hepatocyte function, maintenance of albumin production, and upregulation of other various hepatocyte functions; (4) HGF, excreted by mesenchymal cells or nonparenchymal liver cells, antagonizes the latter process, resulting in support of growth and differentiation of the fetal hepatocytes. The hormone insulin synergistically promotes this effect [27, 29, 31].
In parallel, the percentage of bipotent cells is markedly reduced. At this point, although cells continue to proliferate, most of them are unipotent and irreversibly committed to either the hepatic or cholangic lineage [29-32]. Complete functional hepatic maturation ultimately takes place after birth upon coassistance of HGF, produced by the surrounding nonparenchymal liver cells (sinusoidal, stellate, and endothelial cells) [27, 31]. About half of the active genes tested in the liver are bound by the transcription factor HNF4 alpha and it is suggested that it directly regulates hepatic genes and is an important transcription factor during the differentiation process of ES and iPS cells into mature hepatocytes [34, 35].
It is important to mention that mouse iPS cells exhibit full potential for fetal liver development in embryos derived solely from mouse iPS cells by tetraploid complementation compared to wild-type embryos as indicated by similar levels of hepatocyte marker mRNA and liver specific cell type protein expression levels. In addition, liver Hematoxylin and Eosin (H&E) stained sections presented a similarity in liver morphology between the two [36].

2.1.2 Induction of ES and iPS cell differentiation into hepatocyte-like cells in vitro

The resultant paradigms, taking these cues into account, can thus be broadly grouped in terms of temporal regulation through cytokine addition or spatial regulation using various extracellular matrix configurations. Various configurations for generating hepatocyte-like cells in vitro involve culturing human ES and iPS cells on extra cellular matrix and inducing differentiation with hepatocyte development stage specific factors in order to maximize the yield of cells at each stage which will determine the overall differentiation yield of the process.

As mentioned above, the first step involves the differentiation of pluripotent human ES into endodermal cells and most differentiation paradigms utilize Activin A which mimics Nodal signaling. At the end of this stage, most of the cells lost the expression of the pluripotent marker Oct4 while concomitantly gaining strong expression of definitive endoderm (DE) transcription factors Sox17, FoxA2 as well as GATA4 [36-38]. The later steps of differentiation induce endodermal like cells to differentiate into mature and fully functional hepatocytes while utilizing different ECMs and various factors for about 15 additional days [36-38].

The human ES cells exhibited a similar differentiation efficiency as human iPS cells while using the same differentiation procedure (about 81% albumin positive cells) as well as similar hepatocyte mRNA fingerprints [36] . In addition, cell properties of primary human hepatocytes such as albumin and urea secretion, as well as cytochrome CYP450 activity were similar in vitro between hepatocyte-like cells generated from human ES as well as those generated from human iPS cells, although lower compared to primary isolated hepatocytes (10 fold difference for urea and albumin secretion and 30 fold difference for CYP450 isozyme activities after phenobarbital induction) [38]. While comparing the expression of a series of genes encoding phase I and phase II hepatic enzymes between cadaveric liver samples and hepatocyte-like cells, it was indicated that the level of mRNA was similar between the ES and iPS derived hepatocyte-like cells while lower compared with adult liver samples in most cases [36]. This may indicate that differentiated cells are not mature enough and this step of the differentiation procedure demands further optimization [36, 38].

Human ES and iPS cell derived hepatocytes were shown to contribute to the liver parenchyma in vivo and were stained positive for albumin 7 days after their injection to the right lateral liver lobe of newborn mice, which exemplifies the potential of human iPS cells in regenerative medicine therapeutics [36].

2.2 Differentiation paradigms utilizing tissue engineering

A key requirement for effective tissue engineering is the cellular environment that allows the cells to maintain the functional capacity observed in the in vivo environment. Often the environment mimics some critical aspects of the in vivo setting through proper control of the materials and mechanical properties as well as the chemical milieu.

A consideration with tissue-engineered constructs is the presence of exogenous chemical and mechanical stimuli such as soluble growth and differentiation factors as well as mechanical forces (e.g., cyclic mechanical loading, fluid shear).

2.2.1 Matrix configuration

Culturing cells in various controlled three-dimensional (3D) environments has proven to be a successful differentiation technique. This is thought to be a result of mimicking the *in vivo* histoarchitecture of liver, incorporating cell-matrix interactions as well as soluble cues. Several ECMs are present in liver development. One study compared the effect of using type I collagen, fibronectin, laminin, and Matrigel™ on directing hepatic differentiation. Cells were cultured for 7 days in embyoid bodies (EBs) before being transferred to one of the substrates. This mimics to some extent the liver development stage where endodermal cells migrate into the ECM rich septum transversum. Type I collagen was shown to cause the greatest increase in liver specific genes, with Matrigel™ also showing a beneficial effect[39].

Therefore, one research group utilized a collagen scaffold as a 3D network for hepatic differentiation. Mouse ES cells were cultured in an EB configuration and implanted into a collagen scaffold and cultured in media containing aFGF, HGF, oncostatin M, dexamethasone, and insulin transferrin sodium selenite (ITS). Cells cultured in the 3D configuration showed gene expression of AFP, ALB, glucose 6-phosphate (G6P) and tyrosine aminotransferase (TAT) after 6 days in cultures as opposed to 12 days in an EB configuration alone. However, these cells stained positive for albumin protein, but not for the CK-18 protein. Following implantation into the median lobe of the liver of a nude mouse for 14 days, the cells stained positive for both CK-18 and albumin proteins. This result signifies that cells were not completely matured by the in vitro protocol [40]. While this study showed the advantages of 3D culture, most studies fail to demonstrate a genetic or functional advantage relative to standard monolayer differentiation protocols. Other groups have used 3D cultures to provide a configuration suitable for direct implantation, eliminating the need to remove cells from culture by using biodegradable and/or biocompatible materials. For example, one group used a polyurethane foam spheroid culture to direct differentiation of mouse ES cells. Embryoid bodies were inoculated into a block of polyurethane foam (PUF) to induce the formation of spheroids in the pores of the scaffold. Induction of hepatic differentiation was accomplished by supplementing the media with aFGF, HGF, oncostatin D, dexamethasone, and ITS. Analysis of gene expression showed the expression of endoderm markers transthyretin (TTR) and AFP as well as hepatocyte specific albumin, arginase, and tryptophan 2,3-dioxygenase (TDO) expression. Notably, ES cell derived hepatocyte like cells demonstrated ammonia clearance and albumin secretion rates within the range, albeit on the low end, of those seen for primary rat hepatocytes seeded in the PUF scaffold. However, this method of differentiation is lengthy and immature endoderm markers were still present at the end of the 30 day protocol[41]. Biodegradable polymer scaffolds have also shown to be an effective three-dimensional environment for hepatic differentiation. One study allowed ES cells to form EBs for 5 days before being mixed them with Matrigel™. The cell suspension was then seeded into a rigid polymer network comprised of poly-L-lactic acid (PLLA) and polyglycolic acid (PGA). The cells formed spheroids along the polymer fibers and were cultured for 20 days with dexamethasone, dimethyl sulfoxide (DMSO), FGF4, HGF, oncostatin M, and ITS. After the culture period, the cells showed expression of AFP, ALB, G6P, TTR, and CK-18 and the ability to secrete albumin, uptake low-density lipoprotein (LDL) and store glycogen [42].

Encapsulation of ES cells in alginate has been explored as a method for the control of hepatic differentiation. The capsules allow the diffusion of nutrient, oxygen, and growth factors into the capsules while sequestering the cells. This technique has been widely used in the past to induce stem cell differentiation and maintain hepatocyte function, making it an ideal candidate for controlled hepatic differentiation. For instance, one group encapsulated 5-day-old embryoid bodies derived from mouse ES cells in a 2% alginate solution. The media was supplemented with aFGF, HGF, oncostatin M, dexamethasone, and ITS, similar to those factors previously used by other research groups. RT-PCR analysis determined that this methods results in the expression of endoderm (AFP) and hepato-specific (ALB, Cyp7A1, TAT, TTR, and CK18) genes. The cells produced albumin and urea, but only with growth factor supplementation. If one takes a closer look at the temporal addition of growth factors in these systems, several similarities exist. FGF is usually added at early time points in order to promote differentiation of definitive endodermal cells into hepatic endoderm cells. This is almost always followed by HGF supplementation, mimicking signaling from the

mesenchyme which promotes growth and differentiation of fetal hepatocytes. Lastly, oncostatin M, dexamethasone, and ITS are added, as these factors are known to promote hepatocyte maturation. Sequential supplementation therefore mimics in vivo development. In addition, ES cells are allowed to form embryoid bodies prior to being cultured in a 3D environment. This allows the formation cell-cell interactions known to increase hepatocyte function during development. Although these aforementioned methods yield hepatocyte-like cells from a functional perspective, growth factors are expensive and therefore these approaches are not amendable to scale up for clinical application.

A different approach is the use of cellular encapsulation to control lineage commitment and final maturation of murine ES cells. The novelty of this method lies in the fact that no growth factor supplementation is required to direct hepatic differentiation. This was accomplished through the manipulation cell seeding density and alginate concentration. These two variables dictate the size of cellular aggregates that form within the capsule which in turn direct differentiation. It was found that a 2.0% w/v alginate concentration and a $5x10^6$ cells/mL seeding density were the optimal parameters for hepatic differentiation [43, 44]. Genetic analysis showed the expression of a variety of Cyp450s as well as CK-18. In addition, albumin and urea secretion as well as glycogen storage were shown and reached a maximum around day 20 in culture. This method demonstrates a way to obtain a high yield of hepatocyte-like cells through inducing cell-cell interactions known to upregulate hepatocyte function during development, thus eliminating the need for growth factor supplementation. In addition, the cells can be recovered from the capsule through depolymerization without effecting cell viability. Scalability is also significant, as generating large numbers of cells would simply involve producing large batches of capsules. With all these advantages taken into consideration, this technique is amenable to the mass production of hepatocyte like cells and thus has the potential for clinical utility.

2.2.2 Coculture

A co-culture of ES cells with another cell type present during hepatic development or in the adult liver cell has been shown to induce hepatic differentiation. The supporting cell type directs the ES cells towards the hepatic lineage by introducing cues resulting from soluble factors, cell-cell interactions, or a combination of the two. Thus, this approach is performed by separating the cells with a porous membrane or with the cells in direct contact with one another. Both methods have been shown to induce hepatic differentiation of embryonic stem cells with careful choice of feeder layers. In fact, investigators often develop their own cell lines optimized to drive hepatic differentiation. For example, one group first developed a protocol to differentiate endoderm cells by culturing the cells on Matrigel™ and supplementing the media with activin A and HGF [45]. The endoderm cells were separated by fluorescence-activated cell sorting (FACS), made possible by the transfection of enhanced GFP (EGFP) under the control of an AFP promoter. The purified cells were then further exposed to a co-culture with MLSgt20 cells, a cell line cloned from fetal murine stromal cells experimentally shown to promote hepatic differentiation in ES cells [46]. Combining the results of the two studies, the AFP positive endoderm cells were contact co-cultured with the MLSgt20 cells. The co-cultured cells expressed markers for both immature (GATA4, AFP) and mature (Alb, TAT, TO, CYP3a4/7) hepatocytes at the end of the culture period. The cells also showed the ability to clear ammonia and store glycogen. However, gene expression was not identical to that of adult human liver hepatocytes [47]. A drawback of contact co-culture is that the hepatocyte like cells must be separated from the supporting cell

type after the culture period. To eliminate the need for purification of differentiated cells, one group co-cultured three human liver non-parenchymal cell lines with ES cells using a transwell membrane. Mouse ES cells containing a GFP gene with and albumin promoter were cultured in suspension for 2 days to facilitate embryoid body formation. The EBs were then cultured on a poly-amino-urethane (PAU) coated non-woven polytetrafluoroethylene (PTFE) fabric that allowed the cells to adhere. The media was supplemented with basic FGF and activin A for 3 days. The ES cells were then co-cultured in Matrigel layered transwells with the human cells lines growth-arrested with mitomycin C. Cholangiocytes, liver endothelial, and hepatic stellate cell lines were chosen due to their secretion of soluble factors that have shown to be important for liver regeneration. Cholangiocytes generate IL-6 and TNF-α, liver endothelial cells produce FGF4 and vascular endothelial growth factor (VEGF) and hepatic stellate cells produce HGF. The cells were co-cultured with media supplementation of dHGF and DMSO for 8 days and dexamethasone for the final 3 days of culture. The differentiated GFP positive cells were separated by cell sorting and showed a yield of 70%. These cells expressed both hepatocyte markers as well as endoderm markers, demonstrating that the cells were not fully mature. They also stained positive for albumin and GFP, secreted albumin, and metabolized ammonia, lidocaine, and diazepam. However, albumin secretion and metabolic activity occurred at lower levels than primary mouse hepatocytes[48]. Another example of a non-contact co-culture was demonstrated using cynomolgus monkey ES cells with mouse fetal liver-derived cells to simulate the environment of the developing liver. The ES cells lost pluripotent markers and expressed AFP, ALB, CYP7A1, and HNF4α, an important transcription factor for mature hepatocytes. The cells also stained positively for AFP, albumin, alpha1AT, and HNF4α, as well as for Hep Par 1, an anti-hepatocyte antibody. Functional analysis also showed glycogen storage through Periodic acid-Schiff (PAS) staining as well as ammonia clearance[49].

While the aforementioned co-culture systems utilize non-parenchymal cells or fetal liver cells as a support cell type, the use of hepatocytes as the feeder cell type in co-culture differentiation schemes has also been attempted. Moore et al. examined the effects of co-cultivated hepatocytes on the hepatospecific differentiation of murine ES cells[50]. Hepatocytes co-cultured with cadherin-expressing ES cells markedly enhanced ES cell differentiation toward the hepatic lineage, as demonstrated by hepatic-like cuboidal morphology, heightened gene expression of the late maturation marker G6P in relation to the early marker AFP, and the intracellular localization of albumin. The effect was mediated by cadherin, since it was reversed through E-cadherin blockage and inhibited in control ES cells with reduced cadherin expression. Direct contact co-cultures of hepatocytes and ES cells maximally promoted ES cell commitment towards hepato-differentiation. This study showed that both soluble signaling and cell-cell interaction creates a synergistic effect that drives hepatic differentiation.

Cho et al. developed another co-culture method with hepatocytes[51]. A collagen gel was formed on tissue culture dishes and primary rat hepatocytes were plated after gelation. A thick collagen layer was then deposited on top of the hepatocytes. We have previously shown that this collagen sandwich hepatocyte culture maintains hepatocyte function in vitro. Murine ES cells were then seeded on the thick collagen layer and cultured in this configuration for 10 days. At this stage, the ES cells were removed from the collagen layer by dispase treatment while leaving the collagen sandwiched hepatocytes intact. RT-PCR demonstrated the presence of endoderm markers Foxa2, Sox17, and AFP and the absence of

mesoderm and ectoderm markers. The presence of Foxa2 and AFP were confirmed by immunostaining, and flow cytometry showed that they were expressed in 95% of cells. In addition, the cells proliferated and stopped expressing Oct4, a marker for pluripotency. These results showed that mouse ES cells cultured on top of collagen-sandwiched hepatocytes differentiated and proliferated into a uniform and homogeneous cell population of endoderm-like cells. However, the endoderm cells did not express albumin, signifying that they had not yet committed to the hepatic lineage. To further mature the cells, they were co-cultured for 20 days with fibroblasts due to the in vivo interactions of the endoderm with the mesenchyme during development. The media was supplemented with oncostatin M, ITS, and dexamethasone. The resulting hepatocyte like cells expressed hepatospecific genes albumin, alpha-1-antitrypsin (AAT), CK-8, CK-18, TTR, and CYP2A13 and displayed morphology similar to that of primary rat hepatocytes. Immunostaining demonstrated the presence of albumin and CK-18 while functional analysis showed the cells could synthesize urea. This study showed the generation of a homogeneous population of hepatocyte-like cells from ES cells[51]. However, the drawback of this method is that two separate co-cultures are required to direct differentiation, with one feeder cell being the scarce cell type that we are trying to generate.

2.2.3 Metabolic engineering

A distinguishing feature of adult hepatocytes is the high content of mitochondria and high level of oxidative metabolism. On the contrary, ES cells contain a much smaller amount of mitochondria and produce energy mainly through the glycolytic pathway. Recent studies show that metabolic additives that promote carbon backbone oxidation can be used to help direct differentiation towards the hepatocyte lineage. One such method utilizes sodium butyrate treatment to generate an enriched population of hepatocyte-like cells from embryonic stem cell [52]. ES cells were plated on gelatin and the media was supplemented with DMSO for the first 5 days of culture. Sodium butyrate replaced DMSO for the next 6 days, and cells were replated onto collagen coated or non-coated polystyrene at various time points in order to perform metabolic analysis. Significantly higher levels of urea secretion and albumin positive cells were observed in a 2.5 mM sodium butyrate condition on both substrates. It was also shown that mitochondrial mass increased from days 5-8 in culture, which is characteristic of hepatic differentiation. However, the percentage of albumin positive and high mitochondrial activity cells was still less than mouse hepatoma cells. These results imply that these cells represent an immature hepatocyte phenotype. A subsequent study was conducted in order to further differentiate the cells into mature hepatocyte-like cells [53]. The immature cells were treated with S-NitrosoAcetylPenicillamine (SNAP), a nitric oxide donor. Nitric oxide is known to induce the synthesis of mitochondria, thus possibly facilitating a further increase of mitochondrial mass to levels seen in mature liver cells. After the 11 days of culture conditions that were found to induce partial differentiation using sodium butyrate, cells were replated on day 12 and supplemented with various dilutions of SNAP in DMSO for the next 3 days. A 500 μM concentration of SNAP significantly increased glucose consumption, lactate production, and the percentage of albumin positive cells. From a functional perspective, SNAP treatment also increased urea and albumin secretion as well as cytochrome P4507a1 activity relative to the other culture conditions. These studies demonstrate that simply altering the ES cell's metabolic activity to more closely resemble those of mature hepatocytes is an alternative

strategy to developing hepatocyte-like cells. This novel method circumvents the need for media supplementation with expensive cocktails of growth factors or co-culture with another cell type.

3. Utilizing hepatocyte-like cells

Techniques aimed at obtaining a homogeneous population of hepatocyte-like cells with functional characteristics similar to native hepatocytes from ES cells are rapidly improving. As these methods progress, applications taking advantage of a constant supply of hepatocytes are being developed in parallel. One application is the use of hepatocytes for the treatment of liver failure. With the gap between those on the waiting list for a liver transplant and the organs available for transplantation growing every year, a renewable source of hepatocyte-like cells could potentially alleviate this problem. Bioartificial livers containing functional hepatocyte-like cells could be used to keep patients alive while waiting for a transplant. In addition, decellularizing livers unsuitable for transplant and reseeding them with functional cells could increase the donor pool. In conjunction, these two cutting-edge methods could reduce the number of patients who die on the waiting list. Finally, the important role the liver plays in the metabolic clearance of drugs makes hepatocytes an important research tool for the pharmaceutical industry. By including microfluidic technology, the number heaptocytes needed per assay can be greatly reduced, facilitating high-throughput screening of new chemical entities. This next section will discuss the current state of these applications of hepatocyte-like cells derived from ES cells.

3.1 Decellularized liver scaffolds for implantation

Using hepatocyte-like cells for transplantation is a potentially very exciting application. As mentioned earlier, a large gap exists between the number of patients waiting for a liver transplant and the number of available liver grafts. With this gap growing each year, an alternative source of transplantable livers is greatly needed. Although the differentiation of hepatocyte-like cells from embryonic stem cells has been accomplished through a variety of methods, creating a three-dimensional organ structure is challenging due to the nutrient and oxygen limitations that occur in the engineered tissue. The liver consists of a complex network of extracellular matrix proteins as well as microvasculature critical to organ function. One approach to provide the complex 3D environment of the liver is not to try to recreate it from scratch, but rather to use natural decellularized liver scaffolds. Data show that the native 3D matrix can promote cell engraftment, survival, and sustained hepatic function over time. In addition, by preserving the native microvasculature structure, the risk of ischemic damage or lack of perfusion is minimized. This approach has the potential of utilizing livers unsuitable for transplantation as a source of scaffolds to seed hepatocyte-like cells derived from ES cells, thereby increasing the number of livers available for transplantation.

Using the liver as a scaffold requires that the organ be decellularized while leaving the 3D architecture intact. Following decellularization, the scaffold must be reseeded with the proper cell types engrafting to their appropriate locations within the tissue to promote function. This has been accomplished by perfusing sodium dodecyl sulfate (SDS) solutions through the portal vein of a rat liver for 72 hours [54]. The result was a translucent structure free of cells which preserved the 3D architecture of the liver, Figure 4a. Most notably, the

microvascular tree was preserved, as shown in Figure 4b. Using the scaffold, cells were seeded by injecting 12.5 million cells in 4 doses at 10-minute intervals. This approach resulted in a grafting efficiency greater than 90%, and perfusion of the recellularized scaffold showed 80% viability over 2 days. Since a liver consists of non-parenchymal cells as well as hepatocytes, epithelial cells were also seeded and engrafted in the lining of the vessel. Moreover, epithelial cell seeding did not affect hepatocyte viability, providing proof of concept that other cell types could also be successfully seeded. The scalability of the system was also tested, injecting a total of 200 million cells using the 4-dose approach. This cell total represents 20% of the adult rat liver mass, double the amount required to provide therapeutic benefit.

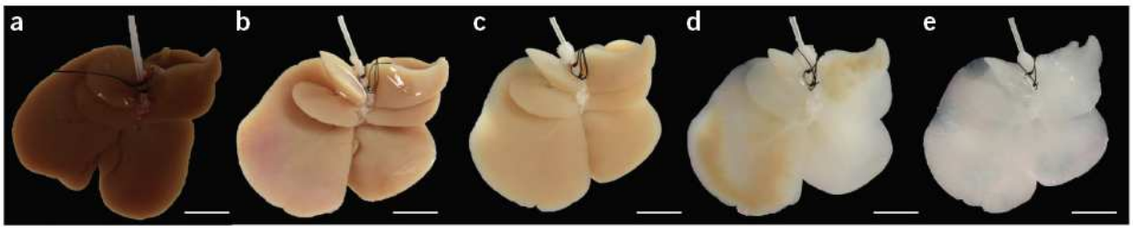


Fig. 4a. Rat liver during the decellularization process. SDS was perfused through the portal vein, leaving a translucent, cell free structure which maintained native liver architecture. From left to right, the images show the liver at 0, 18, 48, 52, and 72 hours of the SDS perfusion, respectively. (Figure taken from [54], Figure 1)

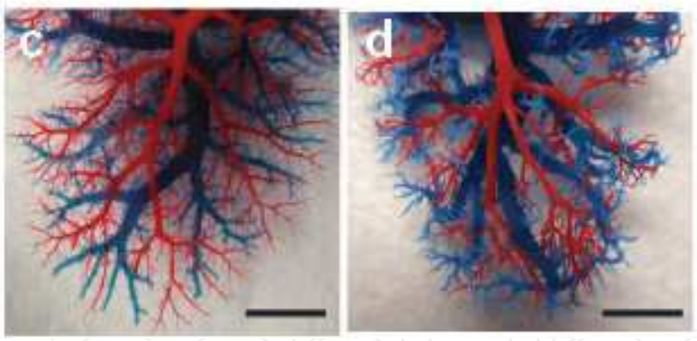


Fig. 4b. Portal (red) and venous (blue) corrosion cast of normal (left) and decellularized liver (right). Comparison of the two shows that the decellularization process preserves the native liver microvasculature. (Figure taken from [54], Figure 2)

Analysis of the reseeded hepatocytes showed gene expression similar to that of long-term stable cultures (e.g. collagen sandwich) of hepatocytes. Immunohistochemical analysis of UGT1a, G6pc, and albumin showed staining at levels comparable to adult livers. Functional analysis showed urea synthesis significantly higher that hepatocyte sandwich and similar albumin secretion rates. However, albumin production was much lower than that of adult rat livers. The recellularized liver was then transplanted into a rat for 8 hours to investigate the effect of shear stress due to blood flow. Transplantation did not affect hepatic function, viability, or morphology in any significant way. Other investigators successfully decellularized a liver and seeded the matrix with both hepatocytes and other epithelial cells

[55]. These studies demonstrate the feasibility of using a decellularized liver matrix as a scaffold for hepatocytes. The inclusion of other non-parenchymal cells and evaluation of the transplant after long periods in vivo still needs to be performed prior to bringing this approach to the bedside. This may further require the development of techniques to derive hepatic nonparenchymal cells from ES cells.

3.2 Bioartificial liver devices

The shortage of donor livers has also prompted researchers to develop bioartificial liver (BAL) devices, **Figure 5**. These devices aim to provide temporary assistance to patients with liver failure while waiting for a donor organ. Early BAL concepts were modified dialysis systems which did not incorporate living cells, [56] and were found to be limited in efficacy. Since the liver provides a host of biochemical processing and detoxification functions that are essential to life, it was thought that an effective device should contain liver parenchymal cells (e.g. hepatocytes). Cells were added to the dialysis systems, and again poor results were obtained, in this case because the bioreactor design itself did not allow for sufficient metabolite transport (especially oxygen), and as a result the cells did not function properly or even survive. Next, a support structure was built that allows for the convective and diffusive transport of plasma metabolites to and from the cells in the device [57]. Many creative operational strategies and designs exist [58]; [59]; [60], ranging from packed bed bioreactors [61, 62], to flat plate bioreactors [63]. It was found that hepatocytes can only

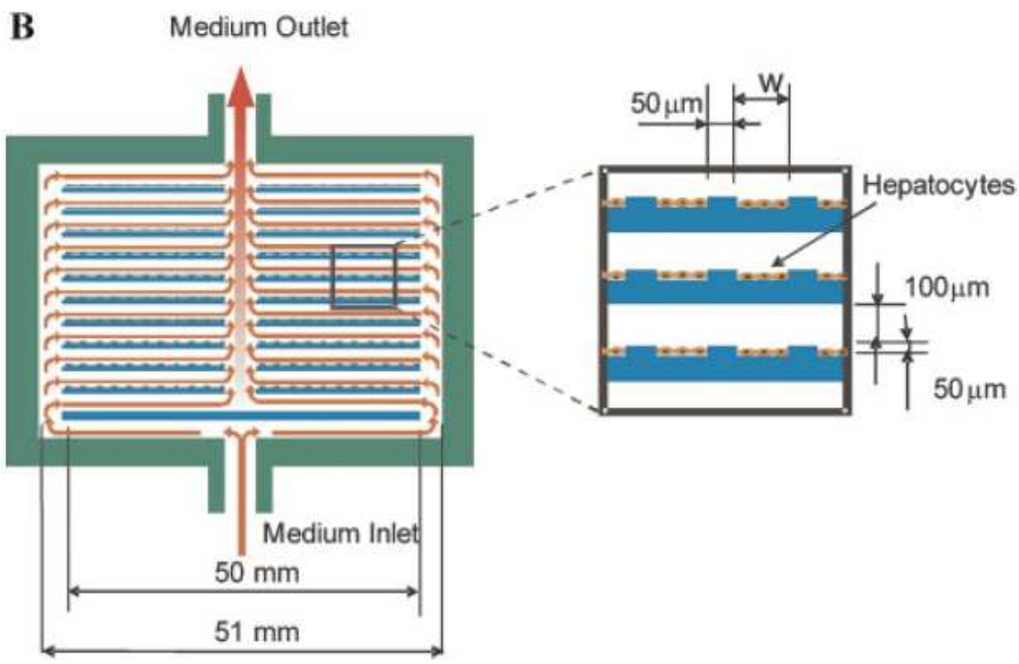


Fig. 5. **Schematic of a radial flow Bioartifical Liver Device.** Red arrows show the direction of medium flow and in blue is the location of the seeded hepatocytes. The design includes patterned microgrooves to decrease shear stress on the seeded hepatocytes. (Figure taken from [67], Figure 1)

withstand low levels of fluid shear stress, therefore in a recent modification of the flat plate geometry, cells were seeded at the bottom of grooves to allow for higher flow rates, thus increasing mass transport within the device, while keeping shear stress at the cell surface to a low level [64, 65].

The high metabolic activity of hepatocytes makes the transport of oxygen a significant technical concern in BAL devices. In fact, transport limitations have led to the loss of hepatic function over time and the failure of many BAL devices based on hollow fiber technology that have undergone clinical trials. To alleviate this issue, semi-permeable membranes have been introduced into devices to increase the oxygen tension within the device. However, a device would require a surface area of approximately 10 m^2 to provide therapeutic benefit [66]. Therefore, the incorporation of an internal membrane oxygenator would create scale up issues. To address these problems, a radial stacked plate bioreactor was developed to increase the surface area to volume ratio of the device. The design was devoid of a semi-permeable membrane and therefore required perfusion at high volumetric flow rates in order to create the necessary oxygen tension to maintain hepatocyte function. Nevertheless, hepatocytes will also lose function above a critical shear stress. Therefore, photolithographically patterned microgrooves were incorporated to shield the cells from the resulting high shear rates required for sufficient convective oxygen transport.

The requirement of high-flow rates and low shear stress is a non-trivial engineering problem. In order to examine the effect of microgrooves, computation fluid dynamics (CFD) was employed to characterize the device. CFD analysis showed that varying width of the microgrooves with the distance from the center of the bioreactor results shear stresses well below detrimental levels throughout the device. Incorporating an oxygenator within the perfusion circuit, design and operating parameters were optimized that provided sufficient convective oxygen delivery and acceptable shear stresses. These critical parameters resulted in 95% viability of hepatocytes under perfusion after 36 hours within the device. In addition, urea and albumin secretion levels were significantly higher than a flat plate bioreactor and comparable to hepatocytes co-cultures with fibroblasts. The device produced 2,100 µg of albumin per day, as compared to $19x10^5$ µg/day required for a therapeutic effect. To be clinical useful, this device would need to be scaled by a factor of 905, which would allow the device to be operated without exceeding the recommended clinical priming volume[67].

As opposed to *ex vivo* bioartificial liver devices, an implantable liver assist device (LAD) has been developed by engineering a hepatic organoid. The implantable construct comprised of a PAU-coated PTFE non-woven fabric covered with a polyethylene-vinyl alcohol membrane layered with a polyester supporting fabric. Based on the method described in Section 2.2.2, human cholangiocytes, liver endothelial cells, and hepatic stellate cells were injected into an LAD coated with FGF-2 to facilitate angiogenesis. The LAD performance was evaluated through implantation into mice after a 50% hepatectomy for 7 days. While LAD with only hepatocytes did not survive, devices with non-parenchymal cells exhibited the formation of organotypic structures in the LAD similar to the liver acinus. Additionally, in vitro analysis showed that the organoid significantly improved ammonia and lidociane clearance as well as albumin secretion relative to device with seeded with hepatocytes alone. This study showed that the inclusion of non-parenchymal cells in liver assist devices has the potential of improving *in vivo* performance.

3.3 In vitro drug screening systems

According to the Pharmaceutical Research and Manufacturers of America (PhRMA) U.S. drug companies spent $62.4 billion on research and development in 2009. Studies indicate that it can cost more than $800 million, of which 80% is spent on clinical trials and development, and will take between 8 and 10 years of development to bring a new drug to market. Among candidate drugs that make it past Phase I clinical trials, 50% fail due to human toxicity and bioavailability issues. Moreover, of all candidate drugs, 90% do not make it through final stages of development. This tremendous attrition rate has not improved in recent years. To curb such costly failures, a significant amount of research has been dedicated to identifying in vitro screening systems; i.e., approaches that can be utilized in preclinical phases of discovery and development that offer greater utility in predicting in vivo subcellular and cellular physiological responses.

Currently, a majority of hepatic in vitro screening assays employed within the field of drug metabolism and pharmacokinetics (DPMK) utilize hepatocytes cultured under fully static conditions. In such assays, hepatocytes are either adhered to the bottom of a microtiter well-plate to which culture medium containing candidate drug(s) is subsequently added; or else the hepatocytes are maintained inside the microtiter well in suspension in the media. The microtiter plate is shaken to facilitate mixing and transport, but there is no means for providing a continuous flow of culture media over the cells.

The lack of continuous flow produces functional limitations in these conventional static systems when compared to microfluidic systems. The presence of flow helps regulate the concentrations of both metabolites and cellular by-products in the immediate vicinity of the hepatocytes; whereas in a static system these concentrations are ever-changing until a saturation/depletion condition is attained through accumulation, uptake, and reaction. In a flow system it is possible to maintain a pseudo steady state (equilibrium) which results in optimal working characteristics. Furthermore, in a static well the system is usually mass transport limited; flow can remedy this by the addition of convective mode of mass transport.

A variety of devices have been developed for this purpose [68-70], and in particular to determine parameters for multi-compartmental physiologically-based pharmacokinetic (PBPK) models, Figure 6. For example, a multi-tissue compartmental device has been developed that incorporates a large liver compartment for the assessment of drug absorption both in the liver as well as in other metabolizing tissue types [71-73]. More specifically, the Hμrel® Corporation has developed a liver-specific microfluidic chip that focuses on liver metabolism, with the possible application to liver toxicity assessment, Figure 7. The Hμrel® device allows for the seeding of various cell types to emulate the *in vivo* components affecting pharmacokinetics. The device consists of separate chambers, each seeded with a specific cell type. Device testing demonstrated that the flow co-culture format in the device resulted in better predictions of *in vivo* clearance rates compared to static cultures and flow-based monoculture models, across a wide range of cytochrome P450s. The reason for increased hepatocyte function when flow is incorporated is not yet known for certain. One possibility is that increased mass transport in the system leads to a thinner boundary layer in addition to faster removal of unwanted by-products, together resulting in an observed increased clearance. Regardless of the mechanism, the Hμrel® device yields an in vitro analogue to PBPK models utilizing minimal amounts of cells and drug, thus facilitating high-throughput screening of new chemical entities.

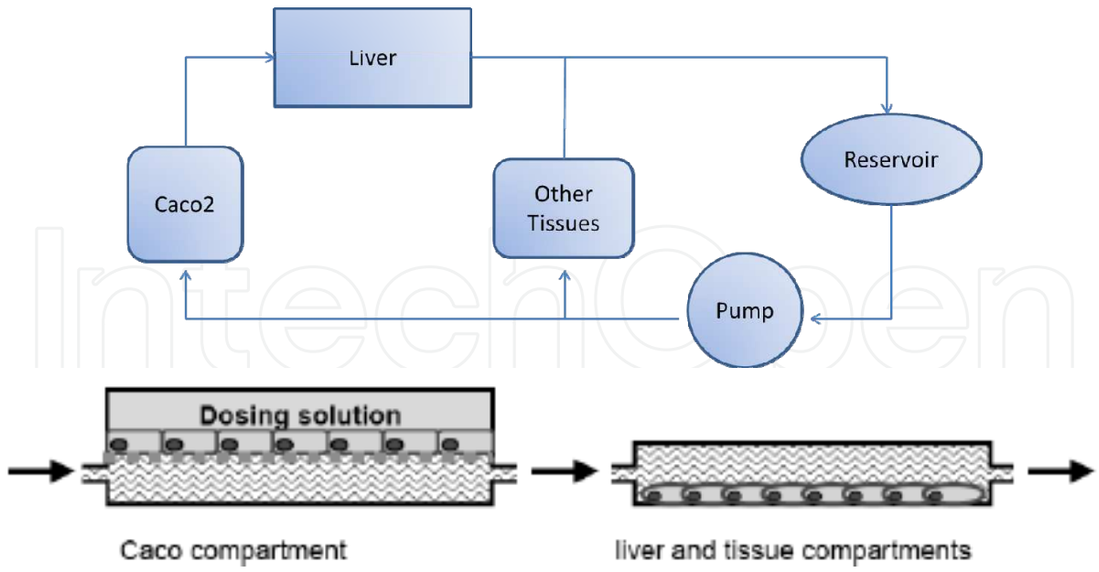


Fig. 6. **Example of multi-compartmental MEMs model utilized to determine PBPK values for use in in silico simulations.** The new chemical entity is dosed on one side of the Caco-2 cells, where it must be absorbed prior to reaching the microchannels. The absorbed drug then passes into the liver for metabolic clearance prior to being re-circulated and reaching the target tissue.

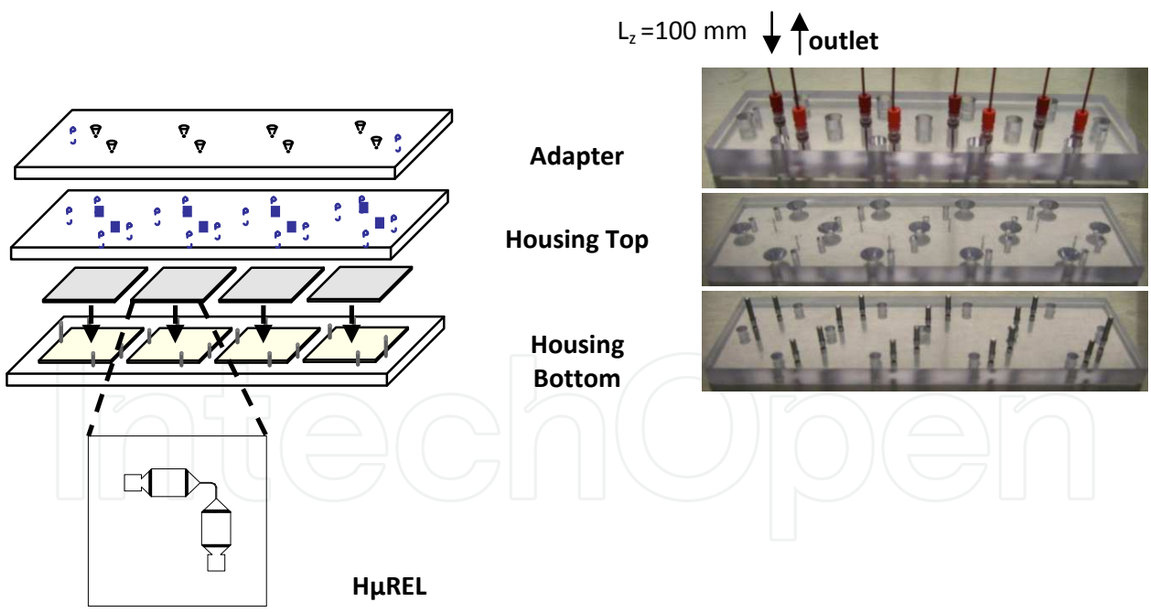


Fig. 7. **Geometry of the static and flow configurations in the HμREL® system.** The assembly of a HμREL® housing set with four biochips. The components of the chip housing and interface are, from the top – adapter, housing top, HμREL® biochips, housing bottom. The complete setup of the HμREL® prototype instrument then involves connecting inlet and outlet tubing to the adapter. The inlet tubing is then fed through a peristaltic pump, and connected to a reservoir that contains the media with the test compound of interest. The outlet tubing is also inserted into the reservoir to complete the recirculation loop. (Figure taken from [75], Figure 1)

4. Future directions

Considerable progress has been made in differentiating ESCs into liver cells; however, current protocols have not yet produced cells that express a completely adult-like mature hepatocyte. In fact, criteria that define what is an acceptable functional human stem cell-derived hepatocyte will need to be established and standardized. ESC differentiation protocols typically do not yield a pure hepatocyte population, and often times sorting protocols are needed. Methods to scale-up such protocols to the therapeutic scale of a human patient will need to be developed. There remains safety concerns (e.g. tumoregenicity) when using ESCs and iPSC for cell transplantation which cannot easily investigated in rodent models and will require further analysis in more "human-like" systems, such as nonhuman primates. On a short-term basis, human hepatocytes derived from ESCs or iPSCs may be effectively used for toxicology studies on xenobiotics as well as drug safety screening. The development of devices that contain such cells for high throughput testing is an important avenue for the future in this area, and special consideration should be taken to make such systems easy to use at the point of care or in the field. The ability to derive cells that are patient-specific provides a unique opportunity to better understand patient variability in their sensitivity to drugs as well as potentially develop individualized patient-specific drug regimens.

5. References

[1] Cho, C.H., et al., *A new technique for primary hepatocyte expansion in vitro.* Biotechnology and Bioengineering, 2008. 101(2): p. 345-356.

[2] Yagi, H., et al., *Long-Term Superior Performance of a Stem Cell/Hepatocyte Device for the Treatment of Acute Liver Failure.* Tissue Engineering Part A, 2009. 15(11): p. 3377-3388.

[3] Maguire, T.J., et al., *Design and Application of Microfluidic Systems for In Vitro Pharmacokinetic Evaluation of Drug Candidates.* Current Drug Metabolism, 2009. 10(10): p. 1192-1199.

[4] Arias, I.M., et al., *The Liver: Biology and Pathobiology.* 1 ed1988, New York: Raven PRess.

[5] Cho, C.H., et al., *Layered patterning of hepatocytes in co-culture systems using microfabricated stencils.* BioTechniques, 2010. 48(1): p. 47-52.

[6] Susick, R., et al., *Hepatic progenitors and strategies for liver cell therapies.* Ann N Y Acad Sci, 2001. 944: p. 398-419.

[7] Forbes, S., et al., *Hepatic stem cells.* J Pathol, 2002. 197(4): p. 510-8.

[8] Wang, X., et al., *The origin and liver repopulating capacity of murine oval cells.* Proc Natl Acad Sci U S A, 2003. 100 Suppl 1: p. 11881-8.

[9] Fausto, N. and J.S. Campbell, *The role of hepatocytes and oval cells in liver regeneration and repopulation.* Mech Dev, 2003. 120(1): p. 117-30.

[10] Zhang, Y., X.F. Bai, and C.X. Huang, *Hepatic stem cells: existence and origin.* World J Gastroenterol, 2003. 9(2): p. 201-4.

[11] Nagy, P., et al., *In vivo infusion of growth factors enhances the mitogenic response of rat hepatic ductal (oval) cells after administration of 2-acetylaminofluorene.* Hepatology, 1996. 23(1): p. 71-9.

[12] LeGasse, E., H. Connors, and M. Al-Dhalimym, *Purified hematopoietic sten cells can differentiate into mature hepatocytes in vivo.* Nature Medicine, 2000. 6(11): p. 1229-1234.
[13] Tan, J., et al., *Immunohistochemical Evidence for Hepatic Progenitor Cells in Liver Diseases.* Liver, 2002. 22: p. 365.
[14] Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. 292(5819): p. 154-6.
[15] Smith, A.G., et al., *Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides.* Nature, 1988. 336(6200): p. 688-90.
[16] Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. 126(4): p. 663-76.
[17] Lister, R., et al., *Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells.* Nature, 2011. 471(7336): p. 68-73.
[18] Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. 131(5): p. 861-72.
[19] Yamanaka, S., *Strategies and New Developments in the Generation of Patient-Specific Pluripotent Stem Cells.* Cell Stem Cell, 2007. 1(1): p. 39-49.
[20] Yamanaka, S., *A Fresh Look at iPS Cells.* Cell, 2009. 137(1): p. 13-17.
[21] Yu, J., et al., *Human Induced Pluripotent Stem Cells Free of Vector and Transgene Sequences.* Science, 2009. 324(5928): p. 797-801.
[22] Feng, B., et al., *Molecules that Promote or Enhance Reprogramming of Somatic Cells to Induced Pluripotent Stem Cells.* Cell Stem Cell, 2009. 4(4): p. 301-312.
[23] McLin, V.A., S.A. Rankin, and A.M. Zorn, *Repression of Wnt/β-catenin signaling in the anterior endoderm is essential for liver and pancreas development.* Development, 2007. 134(12): p. 2207-2217.
[24] Zorn, A.M. and J.M. Wells, *Vertebrate Endoderm Development and Organ Formation.* Annual Review of Cell and Developmental Biology, 2009. 25(1): p. 221-251.
[25] Bossard, P. and K.S. Zaret, *GATA transcription factors as potentiators of gut endoderm differentiation.* Development, 1998. 125(24): p. 4909-4917.
[26] Liu, J.K., C.M. DiPersio, and K.S. Zaret, *Extracellular signals that regulate liver transcription factors during hepatic differentiation in vitro.* Mol. Cell. Biol., 1991. 11(2): p. 773-784.
[27] Kamiya, A., T. Kinoshita, and A. Miyajima, *Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways.* FEBS Letters, 2001. 492(1-2): p. 90-94.
[28] Kamiya, A., et al., *Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer.* Embo J, 1999. 18(8): p. 2127-36.
[29] Kinoshita, T. and A. Miyajima, *Cytokine regulation of liver development.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2002. 1592(3): p. 303-312.
[30] Lemaigre, F. and K.S. Zaret, *Liver development update: new embryo models, cell lineage control, and morphogenesis.* Current Opinion in Genetics & Development, 2004. 14(5): p. 582-590.
[31] Snykers, S., et al., *In Vitro Differentiation of Embryonic and Adult Stem Cells into Hepatocytes: State of the Art.* STEM CELLS, 2009. 27(3): p. 577-605.
[32] Zhao, R. and S.A. Duncan, *Embryonic development of the liver.* Hepatology, 2005. 41(5): p. 956-967.

[33] Sosa-Pineda, B., J.T. Wigle, and G. Oliver, *Hepatocyte migration during liver development requires Prox1.* Nature genetics, 2000. 25(3): p. 254-5.
[34] Battle, M.A., et al., *Hepatocyte nuclear factor 4a orchestrates expression of cell adhesion proteins during the epithelial transformation of the developing liver.* Proceedings of the National Academy of Sciences, 2006. 103(22): p. 8419-8424.
[35] Odom, D.T., et al., *Control of Pancreas and Liver Gene Expression by HNF Transcription Factors.* Science, 2004. 303(5662): p. 1378-1381.
[36] Si-Tayeb, K., et al., *Highly efficient generation of human hepatocyte–like cells from induced pluripotent stem cells.* Hepatology, 2010. 51(1): p. 297-305.
[37] Agarwal, S., K.L. Holton, and R. Lanza, *Efficient Differentiation of Functional Hepatocytes from Human Embryonic Stem Cells.* STEM CELLS, 2008. 26(5): p. 1117-1127.
[38] Song, Z., et al., *Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells.* Cell Res, 2009. 19(11): p. 1233-1242.
[39] Schwartz, R.E., et al., *Defined Conditions for Development of Functional Hepatic Cells from Human Embryonic Stem Cells.* Stem Cells and Development, 2005. 14(6): p. 643-655.
[40] Imamura, T., et al., *Embryonic Stem Cell-Derived Embryoid Bodies in Three-Dimensional Culture System Form Hepatocyte-Like Cells in Vitro and in Vivo.* Tissue Engineering, 2004. 10(11-12): p. 1716-1724.
[41] Matsumoto, K., et al., *Hepatic differentiation of mouse embryonic stem cells in a three-dimensional culture system using polyurethane foam.* Journal of Bioscience and Bioengineering, 2008. 105(4): p. 350-354.
[42] Liu, T., et al., *Hepatic Differentiation of Mouse Embryonic Stem Cells in Three-Dimensional Polymer Scaffolds.* Tissue Engineering Part A, 2010. 16(4): p. 1115-1122.
[43] Maguire, T., et al., *Control of hepatic differentiation via cellular aggregation in an alginate microenvironment.* Biotechnol Bioeng, 2007. 98(3): p. 631-44.
[44] Maguire, T., et al., *Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes.* Biotechnol Bioeng, 2006. 93(3): p. 581-91.
[45] Ishii, T., et al., *Effects of extracellular matrixes and growth factors on the hepatic differentiation of human embryonic stem cells.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 2008. 295(2): p. G313-G321.
[46] Fukumitsu, K., et al., *Establishment of a Cell Line Derived from a Mouse Fetal Liver That Has the Characteristic to Promote the Hepatic Maturation of Mouse Embryonic Stem Cells by a Coculture Method.* Tissue Engineering Part A, 2009. 15(12): p. 3847-3856.
[47] Ishii, T., et al., *In vitro hepatic maturation of human embryonic stem cells by using a mesenchymal cell line derived from murine fetal livers.* Cell and Tissue Research, 2010. 339(3): p. 505-512.
[48] Soto-Gutierrez, A., et al., *Reversal of mouse hepatic failure using an implanted liver-assist device containing ES cell-derived hepatocytes.* Nat Biotech, 2006. 24(11): p. 1412-1419.
[49] Saito, K., et al., *Promoted differentiation of cynomolgus monkey ES cells into hepatocyte-like cells by co-culture with mouse fetal liver-derived cells.* World Journal of Gastroenterology, 2006. 12(42): p. 6818-6827.
[50] Moore, R.N., et al., *Enhanced differentiation of embryonic stem cells using co-cultivation with hepatocytes.* Biotechnology and Bioengineering, 2008. 101(6): p. 1332-1343.
[51] Cho, C.H., et al., *Homogeneous differentiation of hepatocyte-like cells from embryonic stem cells: applications for the treatment of liver failure.* The FASEB Journal, 2008. 22(3): p. 898-909.

[52] Sharma, N.S., et al., *Sodium butyrate-treated embryonic stem cells yield hepatocyte-like cells expressing a glycolytic phenotype.* Biotechnology and Bioengineering, 2006. 94(6): p. 1053-1063.
[53] Sharma, N.S., et al., *Enrichment of Hepatocyte-like Cells with Upregulated Metabolic and Differentiated Function Derived from Embryonic Stem Cells Using S-NitrosoAcetylPenicillamine.* Tissue Engineering Part C: Methods, 2009. 15(2): p. 297-306.
[54] Uygun, B.E., et al., *Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix.* Nat Med, 2010. 16(7): p. 814-820.
[55] Baptista, P.M., et al., *The use of whole organ decellularization for the generation of a vascularized liver organoid.* Hepatology, 2011. 53(2): p. 604-617.
[56] Yarmush, M.L., J.C. Dunn, and R.G. Tompkins, *Assessment of artificial liver support technology.* Cell Transplant, 1992. 1(5): p. 323-41.
[57] Jauregui, H.O., C.J. Mullon, and B.A. Solomon, *Extracorporeal Artificial Liver Support*, in *Principles of Tissue Engineering*1997, R.G. Landes: Boulder CO.
[58] Nyberg, S.L., et al., *Evaluation of a hepatocyte entrapment hollow fiber bioreactor: a potential bioartificial liver.* Biotechnol Bioeng, 1993. 41(194-203).
[59] Kelly, J.H. and N.L. Sussman, *The hepatix extracorporeal liver assist device in the treatment of fulminant hepatic failure.* Asaio J, 1994. 40(1): p. 83-5.
[60] Roy, P., et al., *Analysis of oxygen transport to hepatocytes in a flat-plate microchannel bioreactor.* Ann Biomed Eng, 2001. 29(11): p. 947-55.
[61] Ohshima, N., K. Yanagi, and H. Miyoshi, *Development of a packed-bed type bioartificial liver: tissue engineering approach.* Transplant Proc, 1999. 31(5): p. 2016-7.
[62] Dixit, V. and G. Gitnick, *The bioartificial liver: state-of-the-art.* Eur J Surg Suppl, 1998(582): p. 71-6.
[63] Roy, P., et al., *Effect of flow on the detoxification function of rat hepatocytes in a bioartificial liver reactor.* Cell Transplant, 2001. 10(7): p. 609-14.
[64] McClelland, R.E., J.M. MacDonald, and R.N. Coger, *Modeling O2 transport within engineered hepatic devices.* Biotechnol Bioeng, 2003. 82(1): p. 12-27.
[65] Park, J., et al., *Microfabricated grooved substrates as platforms for bioartificial liver reactors.* Biotechnol Bioeng, 2005. 90(5): p. 632-44.
[66] Park, J., et al., *Microfabricated grooved substrates as platforms for bioartificial liver reactors.* Biotechnology and Bioengineering, 2005. 90(5): p. 632-644.
[67] Park, J., et al., *Radial flow hepatocyte bioreactor using stacked microfabricated grooved substrates.* Biotechnology and Bioengineering, 2008. 99(2): p. 455-467.
[68] Maguire, T.J., et al., *Design and application of microfluidic systems for in vitro pharmacokinetic evaluation of drug candidates.* Current drug metabolism, 2009. 10(10): p. 1192-9.
[69] Novik, E., et al., *A microfluidic hepatic coculture platform for cell-based drug metabolism studies.* Biochem Pharmacol, 2010. 79(7): p. 1036-44.
[70] Chao, P., et al., *Evaluation of a microfluidic based cell culture platform with primary human hepatocytes for the prediction of hepatic clearance in human.* Biochem Pharmacol, 2009. 78(6): p. 625-32.
[71] Park, T.H. and M.L. Shuler, *Integration of cell culture and microfabrication technology.* Biotechnology progress, 2003. 19(2): p. 243-53.

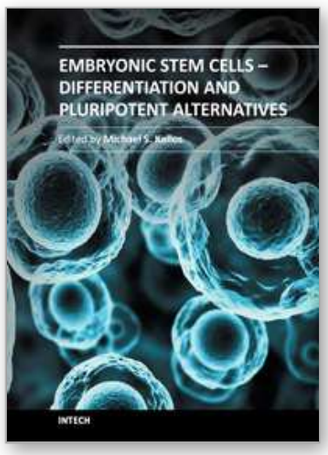
[72] Ghanem, A. and M.L. Shuler, *Combining cell culture analogue reactor designs and PBPK models to probe mechanisms of naphthalene toxicity.* Biotechnology progress, 2000. 16(3): p. 334-45.

[73] Sweeney, L.M., et al., *A cell culture analogue of rodent physiology: Application to naphthalene toxicology.* Toxicology in vitro : an international journal published in association with BIBRA, 1995. 9(3): p. 307-16.

[74] Zorn, A.M., *StemBook. The Stem Cell Research Community*, in *Liver development*, L. Girard, Editor 2008.

[75] Chao, P., et al., *Evaluation of a microfluidic based cell culture platform with primary human hepatocytes for the prediction of hepatic clearance in human.* Biochemical Pharmacology, 2009. 78(6): p. 625-632.

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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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