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The Liver Vascular Bed for Hepatocytes Cell Therapy and Tissue Engineering

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1. Introduction

Liver replacement is necessary for acute and chronic end stage liver failure and for correction of metabolic disorders. Even a temporary liver support is relevant under specific clinical conditions, giving cell based therapy a great potential. The decision on how much liver tissue should be replaced, depends on the degree of liver dysfunction and the chance of the native liver to recover. To minimize complications it is believed that the maximal percentage of engraftment that can be achieved is in the range of 2-5% of the host hepatocytes, whereas 10% are needed for an adequate support in liver failure. Several questions would still rise such as What is the best location for liver cell therapy? What kind of cells should be used? What strategy to increase cell engraftment should be employed? In this review we will discuss the experience gained with intravascular cell transplantation to the liver and future solutions using new technology for biomaterials production. We will expand on our experience with injectable hydrogel scaffold constructs consisting of PEGylated fibrinogen backbone and diacrylate (PEG-DA). The experience with their use for intravascular injection is still limited.

Hepatocyte transplantation is a promising alternative method for the treatment of acute liver failure and also for the treatment of end stage liver disease. Hepatic cell transplantation can be used to temporarily gain time for acutely failing liver to recover, and to restore liver function in the chronic stage until a liver transplant becomes available. Transplanted hepatocytes can serve for the correction of congenital metabolic defects and for the transfer of genes by ex vivo gene therapy. The techniques used for cell transplantation may be implemented for other kinds of cells for therapy, including stem cells and immortalized-hepatocyte- cell lines. Other advantages of cell therapy over whole organ transplantation include the option to perform multiple hepatocyte transplantations in the same patient, and several patients could potentially be treated from one donor at a cost believed to be one tenth that of orthotopic liver transplantation. Studies have been undertaken to improve the methods related to hepatocyte transplantation, which include hepatocyte isolation from donor livers, cell culture propagation of the hepatocytes, hepatocyte preservation, and genetic modification of the hepatocytes to provide liver-specific functions and longevity. In humans, parenchymal hepatocytes isolated from donor livers remain the best cell source for transplantation, and other cell sources, including stem cells, are at the preclinical and early

clinical stages (Puppi et al. 2011). Major problems do not allow this method to be practical and these include the shortage of organs for cell recruitment and limitations in cell preservation. Moreover technical issues still exist. In rodents old reports suggested that transplanted cells into a normal host with normal liver architecture can integrate into the host liver and function for the long run but this was not proven to be the case (Ponder et al. 1991). New problems appeared when human studies were initiated, such as poor initial and long term engraftment and function. It was agreed in a recent consensus, that to obtain sufficient levels of repopulation of liver with donor cells in patients with metabolic liver disease, some form of liver preconditioning would be required to enhance the engraftment proliferation of donor cells (Puppi et al. 2011). Therefore the switch from the animal model to human studies in the set up of acute hepatic failure is not direct and the amount of cells to be transplanted based on animal studies is probably irrelevant to humans. Moreover, the time needed to reverse a failing human liver which is in the range of 3-6 months would be only a few days in the rodent model making issues of cell engraftment and long term function again irrelevant. What seems now to be the right volume of cells (2-5% of the hepatocytes), to be transplanted into the portal vein in the situation of acute hepatic failure in humans, can be later proven to be insufficient (Fisher and Strom. 2006).

2. Which cell to transplant?

The technique of cell transplantation in contrast to whole organ transplantation allows selecting the proper cells for transplantation as discussed in a recent review (Piscaglia et al. 2010). For the purpose of treating the acutely failing organ adult parenchymal cells will be most suitable. These can be harvested from livers denied for transplantation or from non heart beating livers (Hughes et al. 2006; Mitry et al. 2004). Fetal parenchymal cells may be preferred due to their growth potential (Weber et al. 2010). Both sources of cells are of course limited. Harvesting cryopreserved cells may allow using cells when no immediate donor is available (Terry et al. 2007). The use of stem cells from embryonic source or from induced pluripotent stem cells as a source for hepatocytes is still remote from applicability. It may take long periods to expand and differentiate in culture, and the risk of tumor development always exists (Piscaglia et al. 2010). Xenotransplantation is also not applicable even though some experience was gained using porcine hepatocytes on a bioartificial liver device (Soto-Gutierrez et al. 2006). The fear of transferring infection always exists even though the immune response to transplanted cells seems lesser than to a whole organ (Rhim et al. 1994).

2.1 Where to transplant

The liver naturally is the ideal target organ for cell transplantation, in terms of the unique hepatic organization, interactions with non-parenchymal liver cells and biliary drainage. The spleen is also a viable target tissue for transplantation of hepatocytes since it offers the ability to form differentiated chord structures and reform nearly normal hepatic architectures (Mito et al. 1979). Hepatocytes transplanted to the spleen are responsive to liver regeneration stimuli after partial hepatectomy (Aoki et al. 2005). However, the infusion of cells into the portal system by the intra splenic route may be associated with portal vein thrombosis, liver necrosis, hemorrhage and portal and pulmonary hypertension. For

transplanted hepatocytes to engraft into the host liver and remain functionally viable over the long term, the most important outcome for these cells is to translocate from the portal pedicle into the liver microenvironment, as described by several groups (Ponder et al. 1991; Gupta et al. 1999). Another important limiting factor in utilizing either the intraportal or intrasplenic approach is the number of viable cells that can be engrafted without causing complications. It is believed that the maximal percentage of engraftment that can be achieved is in the range of 2-5% of the host hepatocytes whereas 10% are needed for an adequate support in liver failure (Sohlenius-Sternbeck. 2006; Asonuma et al. 1992). The first report on the intrasplenic transplantation via the intrasplenic arterial route was reported on patients with liver failure awaiting liver transplantation (Strom et al. 1999). Despite these complications and limitations, clinical trials of hepatocyte transplantation have been performed without causing any reported fatalities. The peritoneal cavity offers a large space for hepatocyte engraftment, and contact with portal flow but the peritoneal surface does not support long-term attachment and survival of liver cells. Attachment to collagen-coated beads or microencapsulation allowed for only minimal improvement (Selden et al. 2003; Baldini et al. 2008). Co transplantation with non parenchymal cells showed some promise yet, hepatocytes transplanted to the peritoneum or dorsal fat pad do not express genes as those transplanted in the liver (Gupta et al. 1994; Selden et al. 1995). To overcome the problems of intraportal transplantation in the advanced cirrhotic liver with portal hypertension, transplantation into an extrahepatic site is necessary. A strategy that allow repeated extrahepatic infusion of hepatocytes was tried, using a non-immunogenic self-assembling peptide nanofiber. Developed as a three-dimensional scaffold and combined with growth factors, injected into the muscle of small animals, it resulted in improved hepatocytes function, but applicability to humans seems remote (Navarro-Alvarez et al. 2010).

2.2 The microenvironment

Hepatocytes are the major cellular component of a bioartificial liver support system. Although they can elicit multiple functions by themselves, they depend on the cellular network of the non-parenchymal cells. The latter are major regulators of hepatocyte intermediary metabolism, growth and response to injury. Kupffer cells interact with hepatocytes to produce the acute-phase response, i.e., synthesis of C-reactive protein and alpha2-macroglobulin by IL-6. In *in vitro*, when Kupffer cells and hepatocytes are co-cultured, the production of Nitric Oxide is enhanced (Billiar et al. 1990). Hepatocytes interact with hepatic stellate cells (fat storing cells, Ito cells, lipocytes) for the production of cellular matrix *in vitro*. Hepatic stellate cells proliferate, transform into myofibroblast like cells ("activated" hepatic stellate cells) and synthesize large amounts of extracellular matrix components (Collagens, fibronectin, tenascin, undulin, laminin and proteoglycans). Interaction with hepatocytes is mostly important for tissue remodeling. Myofibroblast like cells participate in the recruitment, activation and migration of inflammatory cells at sites of liver injury and play a role in regulating hepatic microcirculation. The endothelial cells that line the blood vessels and the space of Disse, have an important function in cytokine production and the synthesis of other molecules that support the microvasculature and microcirculation. The vascular growth and remodeling is very much dependent on growth factors and extracellular matrix components. A beneficial effect of co-transplantation with

non parenchymal cell has been shown before (Selden et al. 1995). When hepatocytes formed spheroids they kept their function within the matrices for a longer period of time (Lin and Bissell. 1993; Sakai et al. 2010). A recent paper described the beneficial effect for co-transplantation in the peritoneum and the importance of spheroids structure is also stressed (Selden et al. 2003; Hamazaki et al. 2002; Torok et al. 2011). Bioartificial liver support systems incorporating nonparenchymal cells obtained better results in terms of both survival and function of hepatocytes. A method to prepare a biomatrix by a novel, four-step perfusion decellularization protocol using conditions designed to keep all collagen types insoluble, for repopulation, was reported recently (Wang et al. 2011). The various growth factors attached to the extra cellular matrix allowed for better differentiation of stem cells that repopulated the natural bioscaffold.

3. Major growth factors involved in liver growth, regeneration and angiogenesis

The knowledge obtained to date on liver cell growth and development during regeneration is very applicable to the development of liver tissue bioengineering for therapeutic strategies. Liver regeneration after partial hepatectomy is one of the most studied models for tissue regeneration medicine (Michalopoulos. 2010). We have learned that hepatocytes growth factor (HGF), epidermal growth factor (EGF), transforming growth factor α (TGF α) and TNF α /IL6 system are the most potent mitogens *in vivo* (Nakamura et al. 1987; Michalopoulos. 1990). Mainly nonparenchymal liver cells, such as hepatic stellate cells, and endothelial cells produce HGF (Schirmacher et al. 1992). During liver regeneration TGF α is secreted by both parenchymal and non-parenchymal liver cells. TNF α and IL-6 family of cytokines were shown to have an important role in the regenerating liver as shown in knock out mice (Cressman et al. 1996). The proliferative and anti-apoptotic effect of these cytokines takes place only under special conditions such as those existing after partial hepatectomy (Streetz et al. 2000). Major inhibitors such as TGF beta, activin, p21 and p53, help regulate the regeneration process.

Endothelial GFs: fibroblast GF (FGF) and vascular endothelial GF (VEGF) participate in liver regeneration (Assy et al. 1999). FGF is a potent endothelial GF and a primary liver mitogen. In its acidic form, aFGF makes hepatocytes more responsive to EGF and TGF α . The basic form is mitogenic to rat hepatocytes *in vivo* (Baruch et al. 1991; Baruch et al. 1995). bFGF, attached to macroaggregates, enhanced the engraftment of transplanted hepatocytes, by improving vascularization (Perets et al. 2003). FGF is mostly important also for the differentiation of stem cells into definitive endoderm (Kubo et al. 2004). VEGF has a wide range of activities; it increases the endothelial cells' permeability, facilitates monocytes penetration, and enhances synthesis of plasminogen activator and plasminogen activator inhibitor in endothelial cells. VEGF has in addition a vasodilatory effect mediated by nitric oxide. In the liver, receptors are found on endothelial cells and VEGF can be found in a small quantity within liver cells. It was reported that VEGF is released by both host and transplanted hepatocytes during cell entry into the liver plate (Slehria and Gupta. 1998). VEGF is secreted by replicating hepatocytes induce sinusoidal endothelial cell proliferation during regeneration after partial hepatectomy in rats. vWF translocates to sinusoidal endothelial cells during liver regeneration, a change that may have an important role in tissue remodeling during liver regeneration (Baruch et al. 2002).

4. Intravascular cell based therapy and implantable constructs

The most frequently applied hepatocyte transplantation method in humans is the injection of hepatocyte solution into the portal vein. This method, aimed to allow engraftment of hepatocytes within a defected liver, were used by Fox for the first time to treat a girl with Crigler Najar syndrome (Fox et al. 1998; Chowdhury et al. 1991). This method has the problems of insufficient cell survival and engraftment (Chowdhury et al. 1991; Grossman et al. 1995; Raper et al. 1996). Studies concerning cell injection into the spleen showed rapid translocation of transplanted cell through the spleen into the liver (Gupta et al. 1997). By using radio labeled cells it was demonstrated that cells were largely entrapped within the liver vascular bed and not entered the systemic circulation in normal animals (Gupta et al. 1994; Ott and et al. 2002). Cell occlusion is due to mechanical mechanism, because the diameter of hepatocytes is 20-40 μm whereas that of the hepatic sinusoids is 6-9 μm . The sinusoids can probably dilate up to the size of 40 μm . The total volume of extracellular space is believed to be 15% of total liver volume. Due to this size difference, transplanted cells are deposited in the most proximal sinusoids, adjacent to the portal areas of the liver lobule (Gupta. 2002). While the number of cells passing through the sinusoids to the pulmonary circulation is less than 1% in the normal animal, in animals with portal hypertension and portosystemic collaterals even intra splenic injection results in 50% of hepatocytes reaching the pulmonary circulation (Gupta et al. 1993). The vast majority of cells are destroyed instantaneously in the pulmonary capillaries possibly due to shear stress and some other mechanisms. These may include apoptosis and destruction by Kupffer cells secondary to possible ischemia reperfusion injury in this system, needing the use of vasodilators (Sleehria et al. 2002). The transplanted cells occlude the hepatic sinusoids and the portal vein radicles, and portal hypertension is observed immediately. Interestingly the increased pressure is transient as was studied with albumin macroaggregates (Gupta et al. 1994). It is believed that the most common location for transplanted cells to engraft is the liver sinusoidal bed and manipulation to vasodilate the sinusoidal bed to improve engraftment was tried with some success (Sleehria et al. 2002). We believe that once hepatocytes are in the sinusoids they will go all the way through, and those that remain and have the potential to engraft are those stacked in the portal radicles. This explains the very low transplantation efficiency on the one hand but on the other hand target the portal vein radicles as the site needs to be manipulated, rather than the sinusoidal bed. It is this site that probably allow for redistribution of blood after cell transplantation to reduce portal hypertension and to allow for transplanted cell aggregates to be included in the liver tissue. Therefore there is a rationale for using angiogenic growth factors to accelerate this process and to allow better cellular engraftment (Shani-Peretz et al. 2005). We have used for this purpose VEGF and later Heparanase that on top of its angiogenic stimulation is capable to release other growth factors from the injured liver that favor hepatocytes replication (Carmel et al. 2010; Tsiperson et al. 2008).

It is quite clear that isolated cell transplantation by itself would not be enough and cell based strategies for temporary support systems for both extracorporeal and intra corporeal devices need special constructs (Navarro-Alvarez et al. 2010; Allen and Bhatia. 2002b). These devices include hepatocytes or hepatocytes derived cell lines in order to process patient plasma in the case of extracorporeal systems. Implantable tissue engineered constructs have also been developed for the purpose of long-term liver support (Allen and Bhatia. 2002a).

Construction of thicker tissues, was not possible due to limited diffusion of nutrients and oxygen within the engineered cell mass. It is known that cells can only survive within an area close enough to a source of nutrients and oxygen (Folkman and Hochberg. 1973; Rouwkema et al. 2008). Various techniques in polymer biochemistry and scaffold design to mimic the structure of this vascular network in engineered tissues were used. Strategies have included the co seeding of endothelial cells that spontaneously form capillary-like networks, and the engineering of branching channels to mimic the vascular tree.(Wang et al. 2011; Kaihara et al. 2000). In addition, angiogenesis was induced in within engineered tissues by incorporating angiogenic peptides and growth factors into scaffolds and by engineering cells used in the organ constructs, to express these factors. However, these efforts have fallen short of producing scaffolds that contain a vascular tree with centralized inlet and outlet vessels that are suitable for transplantation and capable of nutrient and gas exchange. (Borselli et al. 2010). Scaffolds were fabricated from synthetic material that allowed micro-patterning of vascular tree-like structures. When growth factors are used alone, they tend to create only a microvasculature consisting of small and fragile capillaries, in a time consuming process, and therefore this technique is only applicable for the engineering of smaller size tissues (Perets et al. 2003).

Development of implantable constructs with vasculature, remains a complicated challenge in terms of finding solutions for, the high metabolic rate of the liver cells, the need for unimpeded transport for large macromolecules and the need for cell-cell interaction (Dixit and Gitnick. 1995). In this respect, adult hepatocytes must interact with the various biomaterials, a process which is poorly understood and one of the key challenges to overcome in this field. Various hepatic tissue engineering approaches have been explored including the attachment of hepatocytes to microcarriers and encapsulated hepatocytes spheroids allowing for immunological protection and cell interaction (Demetriou et al. 1986). Co transplantation is easier in within microcapsules that also have the advantage of immunological protection. The preferred site so far is the peritoneum that can accommodate large volume (Perets et al. 2003; Ambrosino et al. 2003; Teng et al. 2010; Shi et al. 2009; Mooney et al. 1997). Absorbing cells on biodegradable synthetic scaffolds to allow control over scaffold chemistry, and space for cell interaction and vascular growth was used (Perets et al. 2003; Mooney et al. 1997; Lee et al. 2009). A significant advancement in the field of bioscaffold design has been the utilization of decellularized liver tissue creating a three-dimensional scaffold for tissue engineering strategies (Baptista et al. 2011). This study demonstrated that human liver cells can be seeded through the portal vein of a liver bioscaffold, and can be maintained in a specialized bioreactor with constant culture medium perfusion up to one week. Progressive human liver tissue formation was documented, as well as liver-associated functions. Widespread cell proliferation inside the bioengineered liver tissue with low cell apoptosis was also observed. These studies showed the possibility of seeding these bioscaffolds with liver cells from animals, but the possibility of generating functional human hepatic tissue is still in question.

Cell encapsulation techniques their advantages and disadvantages are reviewed elsewhere (Hernandez et al. 2010). Recently, a biomaterial system developed that takes advantage of the biological properties of natural extracellular matrix proteins combined with precisely controllable properties of synthetic polymers for making scaffolds for tissue engineering (Almany and Seliktar. 2005; Seliktar. 2005). The biosynthetic hybrid biomaterials are ideally suitable for hepatic tissue engineering in that both the structure and function of the

construct can be exactly regulated by the compositional alterations to the biological and synthetic constituents of the material (Dikovsky et al. 2006; Underhill et al. 2007). These hybrids are made from extra cellular matrix proteins such as fibrinogen, collagen and albumin, while the synthetic polymer is made from polyethylene glycol (PEG) (Gonen-Wadmany et al. 2007). The PEG constituent is biocompatible with liver cells and already received FDA approval. The PEGylated protein hydrogels have already been applied successfully in orthopedic applications and their biocompatibility has been established with 5-week implantation studies in rats. An additional advantage of the PEGylated protein approach is that the biomaterials are polymerizable using photo-initiation, which provide the ability to directly inject the biomaterial precursors solution together with hepatic cell suspension into the body and polymerize the construct in situ (Shapira-Schweitzer and Seliktar. 2007). The combination of PEG and extra cellular matrix proteins in a PEGylated protein hydrogel system represents an advanced approach in scaffold design.

These polymers have the advantage of size, shape and cell number control. Taking this advantage we have recently formed microcapsules at the size of 200-600 μm (Fig. 1). This size is injectable through a 25G syringe through the portal vein of rats. The injected microcapsules can protect groups of hepatocytes from shear stress and from passing through the sinusoids. These particles are stacked in the portal radicle allowing eventually for a group of cells to engraft into the host liver (Nayshool et al.).

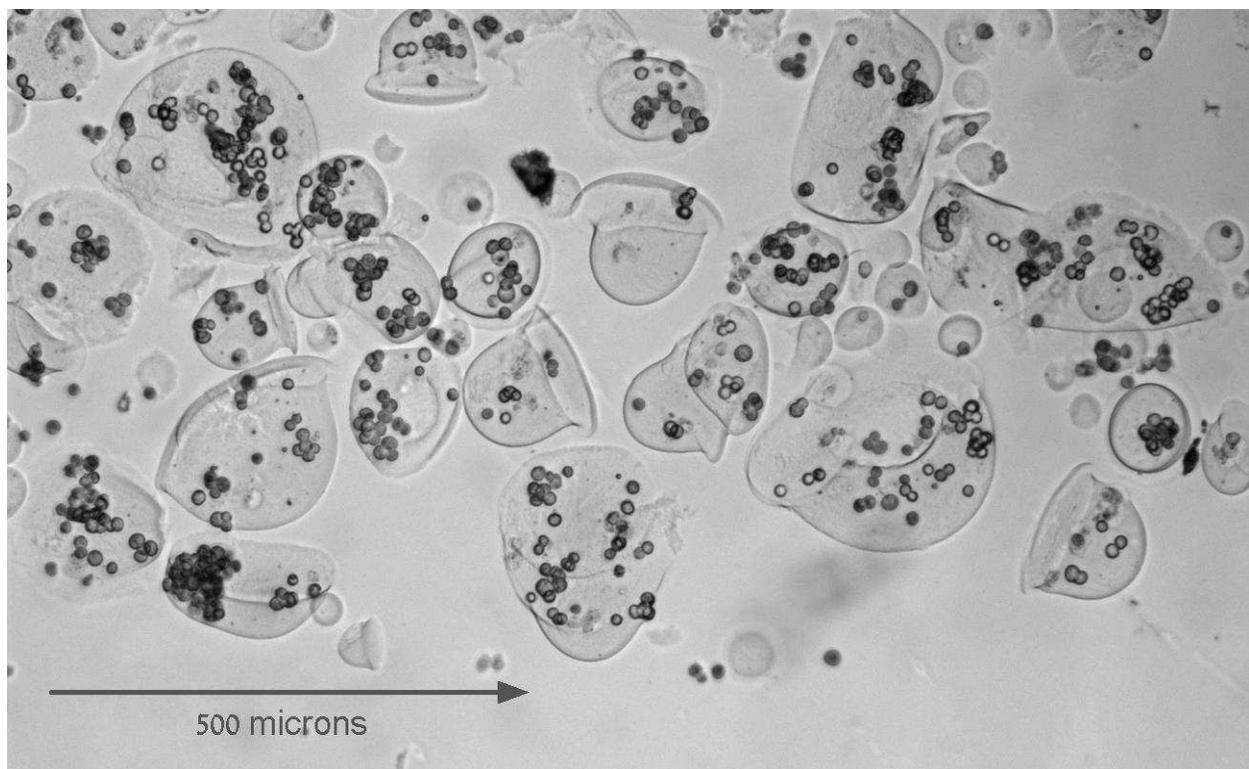


Fig. 1. HUH7 cells encapsulated in a PEG-Fibrinogen hydrogel scaffold. Radius of microcapsules 100-200 micrometers

In the long struggle against liver failure no one method would be most favorable. The strategy may be to employing different methods in the same individual and this would need

the optimization of each one of them. The intravascular route for encapsulated cells is a promising method, solving the problem of shear stress protection, oxygen and nutrient supply, and allow for planning ahead the cell type mixture for transplantation. Still, portal hypertension remain a problem that would need to be resolved.

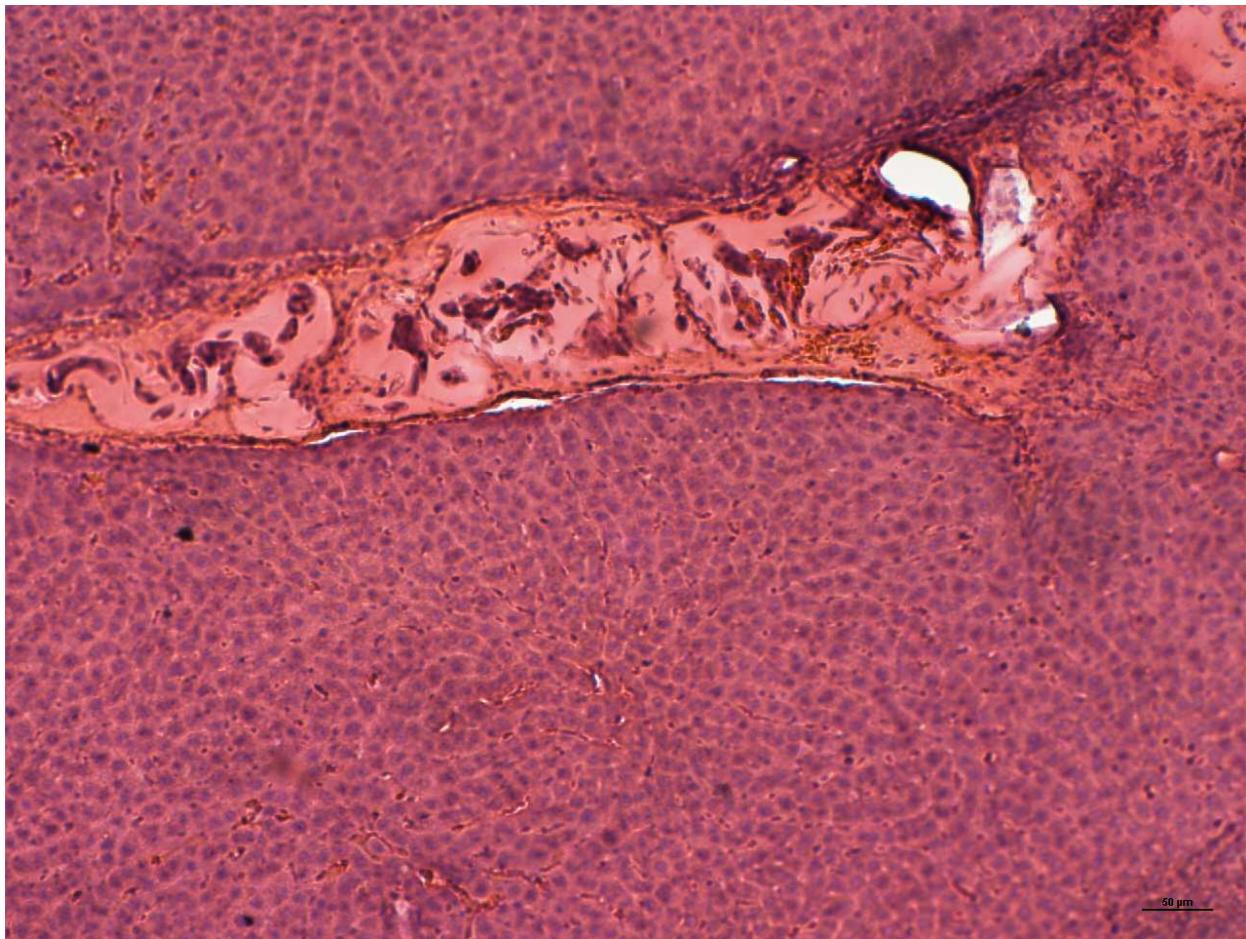


Fig. 2. HUH7 cells encapsulated in a PEG-Fibrinogen hydrogel scaffold in a portal radicle (H&E X40)

Disadvantages	Advantages
Portal hypertension	Natural microenvironment
Portal vein thrombosis	Linkage to bile system
Distorted tissue in acute failure and cirrhosis	Relative large vascular space
Cells bypass to the lungs	Favorable regenerative tissue
Insufficient space	
Coagulation defects	

Table 1. The liver as the target

1. Use of Vasodilators.
2. Combined use of angiogenic and proliferative growth factors.
3. Control for the size of particles.
4. Co-transplantation with non parenchymal cells.
5. Liver preconditioning (partial hepatectomy, portal vein thrombosis, irradiation).
6. Control the size of cell constructs

Table 2. Strategies to improve cell engraftment within the liver

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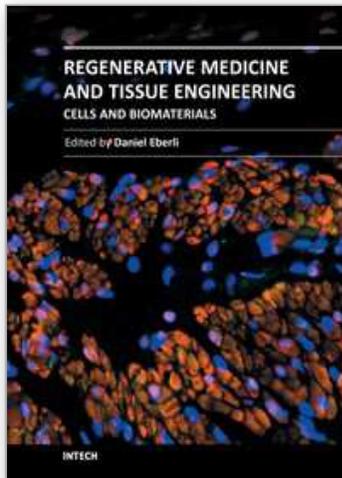
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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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