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Cell Based Therapy for Chronic Liver Disease: Role of Fetal Liver Cells in Restoration of the Liver Cell Functions

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1. Introduction

Liver is the largest organ in the human body and it functions like a metabolic factory. Disruption of its anatomical structure, which is often caused by excessive fibrosis of the extracellular matrix (ECM), when left unattended, can lead to cirrhosis of the liver and cause permanent and irreversible damage to its organization and function, with fatal consequences. Liver cirrhosis, which is generally the end result of chronic liver disease (CLD), can be caused due to many etiological reasons including (a) long term infections with hepatitis B and C viruses, (b) uncontrolled alcohol abuse, (c) excessive exposure to metabolic products of metals like iron and copper (d) autoimmune inflammation of the liver (e) nonalcoholic fatty liver disease (NAFLD) and (f) nonalcoholic steatohepatitis (NASH) (reviewed in [1]). Histo-pathologically, a hallmark of liver cirrhosis is the abnormal production and storage of collagen molecules in the ECM, formation of a scar tissue that replaces normal parenchyma and blockage of the portal flow of blood through the organ, thus affecting normal hepatocellular activity and ultimately total loss of liver functions [2, 3]. Cirrhosis of the liver in early stages is largely asymptomatic, therefore remains undetected by physical examination and other available tests. Diagnosis of fibrosis at early stages and prevention of its progression to cirrhosis is a very important factor in the management of the disease. Among the different options available for treatment, this review would focus on cell based therapy for liver cirrhosis with a special attention on the challenges and procedures of using human fetal liver cells. Methods to improve the clinical application of cell and tissue imaging of liver in the management of cirrhosis would also be discussed, briefly.

2. Treatment options for liver cirrhosis

The established choices of treatment for cirrhosis are very limited and in most cases withdrawl of the underlying causative agent is used as the first line of treatment. Anti-viral

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therapy and biochemical modulation of liver metabolism are some of the classical treatment strategies but more recently several cell therapy based options have been used albeit many are still experimental and limited to preclinical studies. Some of the approaches for treatment of cirrhosis are discussed below.

2.1 Liver transplantation

Liver transplant is considered to be one of the best curative treatment solutions available for advanced liver cirrhosis. However, the treatment procedure carries operative risk, is expensive, requires life long immunosuppression, and there is also a risk of graft rejection that requires a re-transplantation of the organ. One of the major limitations of liver transplantation is the availability of donor liver. There is a constant rise in number of patients on the waiting list for donor liver and an acute shortage on the availability, which is the leading cause for the increase in morbidity and mortality. This gap in the demand and supply of a donor liver tissue is partially filled by obtaining liver tissues from living donors and doing auxiliary liver transplantation. However, while opting for this procedure the possible risk to the donor and the benefits of the recipient must be considered. In this scenario, there is a clear need to look for alternative strategies for the therapy of end stage liver diseases that would be more effective and safe in reducing the tissue scarring of fibrotic livers and in redemption of normal liver function.

2.2 Cell based therapy of liver diseases

Cell based approaches for treatment of liver diseases offer novel but challenging alternatives to liver transplantation. Several types of stem and progenitor cells have been explored for their possible use in this field. Cell based therapies could be initiated by either (a) the activation or mobilization of autologous stem cells to the site of injury or (b) by the infusion of heterologous (or autologous) stem and progenitor cells from different sources.

2.2.1 Activation of autologous regenerative cells

The therapeutic role of autologous regeneration by resident cells in the liver or by mobilized cells from the bone marrow has a significant role to play in the auto regeneration of the normal tissue following liver injury. The failure of these mechanisms leads to the activation of degenerative cascades in the liver such as necrosis, cell death and abnormal accumulation of collagen. The autologous cell based therapies utilise the reactivation mechanisms where either the non responding resident cells in the liver are activated or fresh cells from the bone marrow are mobilised to the site of injury.

2.2.1.1 Activation of resident regenerative cells in the liver by injectible growth factors

In a cirrhotic liver the normal architecture is completely disrupted by abnormal accumulation of ECM components that block the vascular supply leading to the death of liver parenchyma which contributes to the decrease in liver function. The liver has a spontaneous regeneration potential to compensate for this loss of liver parenchyma by division of the existing hepatocytes and hepatic progenitor cells (HPC). However due to extensive scarring of tissue the regenerating cells are prevented from regaining their normal function. It is proposed that ECM remodelling can lead to resolution of this blockage and

reactivate the resident regenerative cells in the affected area. Earlier work on ECM remodelling by using matrix modifying factors such as hepatocyte growth factor (HGF) have shown prevention and/or regression of fibrosis in animal models of liver and pulmonary injury [4]. Administration of human rHGF or gene transfer of human HGF to rats with hepatic fibrosis/ cirrhosis caused by di-methyl-nitrosamine prevented the onset and progression of hepatic fibrosis/cirrhosis [5, 6]. Some of the studies have revealed that HGF mediates this process by directly antagonizing the pro-fibrotic actions of Transforming growth factor (TGF)-β1 [7, 8]. In addition to these molecules, studies on basic fibroblast growth factor (b-FGF, FGF-2), in animal models, has shown their participation in tissue regeneration, angiogenesis and in wound healing processes [9]. A recent report has shown that activation of HPC might be linked up with ECM remodelling. Degradation of collagen I and subsequent laminin deposition seem to be important prerequisites for HPC activation and expansion [10]. Based upon these results it appears that a better understanding of the factors that govern HPC proliferation and the resultant ECM changes would provide clues to improve regeneration of liver cells in chronic liver disease.

2.2.1.2 *In situ* mobilization of bone marrow cells to the liver

Liver cirrhosis is associated with an intermittent mobilization of different types of bone marrow cells that are committed to differentiate to hepatocytes [11]. This process could be triggered either spontaneously upon liver injury or by administration of growth factors that could stimulate the migration of stem cells from the bone marrow into peripheral blood. Granulocyte-colony stimulating factor (G-CSF) is one such mobilizing agent that is receiving considerable attention recently in the field of liver therapy. Several studies have indicated that G-CSF may be effective in mobilizing bone marrow cells into the peripheral blood that contribute to liver repair [12, 13]. In rats G-CSF was shown to contribute to liver repair in a double mechanism by increasing the bone marrow derived liver repopulation, and also by activating the endogenous oval cells, that express G-CSF receptor (G-CSFR) [14].

In a clinical trial, 8 patients affected by severe liver cirrhosis were administered G-CSF and the treatment was well tolerated in all the patients during a follow-up of 8 months, and mobilization of bone marrow stem cells co-expressing epithelial and stem markers was noted [15, 16] . Two independent studies on a group of 24 patients and 18 patients [17, 18] with severe liver cirrhosis resulted in a dose-dependent mobilization of good CD34+/CD133+ bone marrow stem cells and proved that the procedure was safe, but did not achieve any significant clinical improvement. Treatment with G-CSF was associated with the induction of HPC proliferation within 7 days of administration [19]. In a recent report G-CSF based mobilization of bone marrow cells was used successfully to treat patients even with acute on chronic-liver failure (ACLF), and a significant recovery in the clinical condition was noted [20]. Use of G-CSF could thus promote the *in situ* mobilization and regeneration of the liver tissue without excessive intervention and is slowly gaining its importance in the field of liver therapy.

2.2.2 Infusion of therapeutic cells

The discovery of stem cells has revolutionized the field of medicine offering potential options for the management of various chronic disorders. Different types of stem and progenitor cells from various sources are available with a broad potential for differentiation and application in tissue regeneration and newer sources are being explored. Several clinical

studies have been reported and reviewed where regenerative cells had been infused for the purpose of liver therapy and many other studies are currently in progress.

Based on the donor tissue source and the differentiation potential of cells the results on the therapeutic efficacy of several cell types has been described below.

2.2.2.1 Infusion of adult hepatocytes and Bio-artificial Livers (BALs)

Adult hepatocytes are the fully mature functional cells of the liver that are highly specialized with the ability to divide and are an ideal source for transplantation. Though hepatocyte transplantation has been recognized as an attractive option for the management of metabolic liver disease some 35 years ago [21], lack of availability of livers for cell isolation, difficulty in expansion of hepatocytes *in vitro a*nd their sensitivity to freeze-thaw are major limitations for their routine use in cell therapy.

Allogeneic primary hepatocytes isolated from cadaver livers and infused via the splenic artery or the portal vein showed an improvement in the clinical condition [22, 23, 24]. Many preclinical studies and clinical applications of this technique have been made to cure metabolic liver disorders and end-stage liver diseases [25]. In most instances, hepatocyte transplantation has been able to grant a clinical improvement for up to 12 months [26].

Implantable hepatocyte-based devices and extra corporeal liver assist devices represent another alternative for the treatment of end-stage liver disease [27]. BAL devices are intended to support the failing functions of the organ, and include both a biological component (parenchymal cells) and an artificial scaffold serving as an interface with patient blood or plasma. Two main clinical trials have evaluated the efficacy of liver assist devices in patients with fulminant liver failure. Owing to the difficulty in supply of human hepatocytes, the devices included either purified pig hepatocytes (HepatAssist) or cell lines derived from liver cancer cells (ELAD) [28, 29]. Both trials failed to demonstrate a beneficial effect on survival. In the future, such devices are likely to re-emerge as a result of new technologies that allow growth and differentiation of large amounts of liver cells *in vitro* from stem/precursor cells. However, BALs will have to challenge cost-effectiveness in comparison with more convenient artificial devices.

2.2.2.2 HPC's and fetal liver cells (hepatoblasts)

Isolation, expansion and differentiation of adult HPCs to functional hepatocytes has been described by several workers [30, 31, 32], however identification of a specific marker of HPCs still remains a challenge. Clinical studies on the repopulation of the human liver by HPCs are still awaited.

Compared to the adult HPCs, fetal liver progenitor cells are highly proliferative, less immunogenic and more resistant to cryopreservation. The human fetal liver, between 10 and 18 weeks of gestation, contains a large number of actively dividing hepatic stem and progenitor cells that are termed as hepatoblasts. These are bi-potent cells , that can give rise to both hepatocytes and bile duct cells; they co-express hepatocyte markers (such as albumin, α-fetoprotein (AFP), α-1 microglobulin, glycogen, glucose-6-phosphatase (G-6-P) and Hep-Par-1) and biliary markers, for example, gamma glutamyl transpeptidase (GGT), dipeptidyl peptidase IV (DPPIV), cytokeratin (CK)-19 and Das-1-monoclonal antibody-reactive antigen, [33, 34]. These markers are expressed throughout the second trimester and thus offer opportunities to isolate and study large numbers of progenitor cells from abortuses.

2.2.2.3 Haematopoietic Stem Cells (HSCs) and Mesenchymal stem cells (MSCs) from the bone marrow

HSCs are committed progenitor cells of the bone marrow and can be extensively expanded without loss of pluripotency. The bone marrow is an important source of autologous HSCs, and MSCs [35, 36]. Infusion of unsorted autologous bone marrow stem cells through the portal vein and hepatic artery in patients with cirrhosis, showed an improvement in Child-Pugh score and albumin levels [16, 37]. A significant increase of liver function in cirrhotic and hepatocellular carcinoma patients was observed following autologous bone marrow stem cell transplantation prior to surgery [38]. However, use of unsorted bone marrow stem cells or MSCs must be treated with caution as these cells can also differentiate into myofibroblasts which are the scar forming cells of the liver [39]. Recent data also provides evidence to this in a rodent model where use of whole bone marrow as cell therapy led to the worsening of liver fibrosis [40]. Macrophages which are the cells of haematopoietic origin, are known to play a critical role in regulating liver fibrosis in murine models [41] and a single intra-portal administration of macrophages has recently been shown to reduce fibrosis in a murine model of liver injury and increase regeneration [40].

MSCs and HSCs can also be isolated from other tissues such as peripheral blood, adipose tissue, umbilical cord blood (UCB) and placenta [42]. Adipose tissue derived MSCs have a good proliferative capacity *in vitro* and *in vivo* and can differentiate into hepatic cells [43-46]. The placenta and UCB are also important sources of young MSCs and HSCs that can be obtained without invasiveness or harm to donor and provide no ethical barriers for basic and clinical applications [47, 48]. Recently UCB cells were used on a diverse group of end stage cirrhotic patients with an improvement in the life span in these patients [49] raising a hope on the use of these cells for liver cell therapy.

MSCs and HSCs from all sources display a high degree of plasticity giving rise to a wide range of phenotypes, including hepatocyte-like cells. The low immunogenic property of these cells have shown promising results with the use of these cells in *in vitro* studies and clinical trials [50-55].

2.2.2.4 Embryonic and Induced pluripotent stem cells (iPSCs)

Embryonic stem cells (ESCs) derived from the inner cell mass of 5-6 day old embryos have the advantage of being able to proliferate in an unlimited fashion and might constitute an easily available source to obtain a large number of transplantable cells for regenerative treatments. By manipulating the factors responsible for maintaining the undifferentiated state of these cells and by exposure to appropriate growth factors *in vitro*, ESCs can be directed towards the hepatic lineage. ESC-derived hepatocyte-like cells were able to colonize the injured liver and function as mature hepatocytes without teratoma formation in several animal models of liver disease [26, 56, 57]. Due to the propensity of these cells to form both malignant and non-malignant tumors, caution has to be still exerted for their use in transplantation. The ethical issues regarding the use of human embryonic stem cells will also have implications for their clinical use.

Induced pluripotent stem cells offer a solution to this ethical concern and the risk of rejection related to embryonic stem cells. iPSCs are generated *in vitro* by genetic reprogramming of

adult somatic cells with certain factors, to form pluripotent stem cells with embryonic-like differentiation potential [58]. Hepatocytes derived from iPS cells have a reasonable synthetic and metabolic capacity [59], and seem to be similar to cells derived from ES cells [60-62]. However, the same concerns of tumor formation or reversion to more primitive state with uncontrolled expansion within the recipient still remain. In spite of these limitations iPS-derived hepatocytes are a very promising population for cell therapies in hepatology.

3. Management of end stage liver disease with fetal liver cells

Hepatic progenitors derived from the livers of electively aborted fetuses of 5 to 20 weeks gestation are generally designated as "multipotent." Fetal cells seem to have an edge over embryonic stem cells in that, being less versatile, they may not form teratomas and exhibit an important property of being less immunogenic by the little to no expression of the Class II HLA marker on their surface, which otherwise can trigger a rejection reaction [63]. Thus, tissue matching that is a prime requirement in blood transfusions, organ transplants, and allogenic adult stem cell transplantation is not necessary when transplanting these cells.

3.1 Hepatic progenitors in developing liver

The developing human (and also murine) liver during early to mid gestation has been shown to comprise stem and progenitor cells that can give rise to the different adult cell types. The developmental plasticity of fetal liver parenchymal cells, at this stage of organ development, makes them a very good source for cell therapy. During the first trimester of fetal development, the liver serves as the site for hematopoiesis and later during the mid-gestation the hematopoietic cells migrate to the bone marrow and the liver starts functioning primarily as a hepatic organ (Figure 1). In the first trimester, human fetal liver is the site of synthesis of progenitor and stem cells of many lineages but during the second trimester, markers for hepatoblasts continue to be expressed but the expression of markers for other lineages is reduced [63, 64]. This offers an opportunity to isolate and study large numbers of hepatic progenitor cells without haematopoietic potential during the second trimester of gestation. It is interesting that hepatic progenitor cells isolated from human fetal liver proliferate for several months and retained their normal karyotypes, thereby indicating a strong telomerase activity in them [65].

3.1.1 Cell surface and intracellular markers for progenitor cells

Many different markers have been used for the identification of progenitor cells. Bi-potential progenitors have been isolated from fetal mouse liver in a number of studies. Petersen et al have established that a Thy-1+ve cell population within the rat fetal liver also expressed CK-18, a hepatocyte marker [66]. Hepatic progenitor cells have also been reported to express c-kit, a hepatic stem cell marker, along with CD34 and Thy-1 [67]. In a study from our lab, CD34 cells from human fetal liver were found to be expressing hepatic and biliary markers as well [64]. In another study with human fetal liver cells, Thy-1$^+$ (a haematopoietic marker) cell populations were isolated that were found to be positive for progenitor (CD34, c-kit, CK14, M2PK, OV6), biliary (CK19) and hepatic (HepPar1) markers, revealing their progenitor as well as hepatic and biliary nature [68]. Expression of these specific markers by each cell type is used for the isolation of progenitor cells.

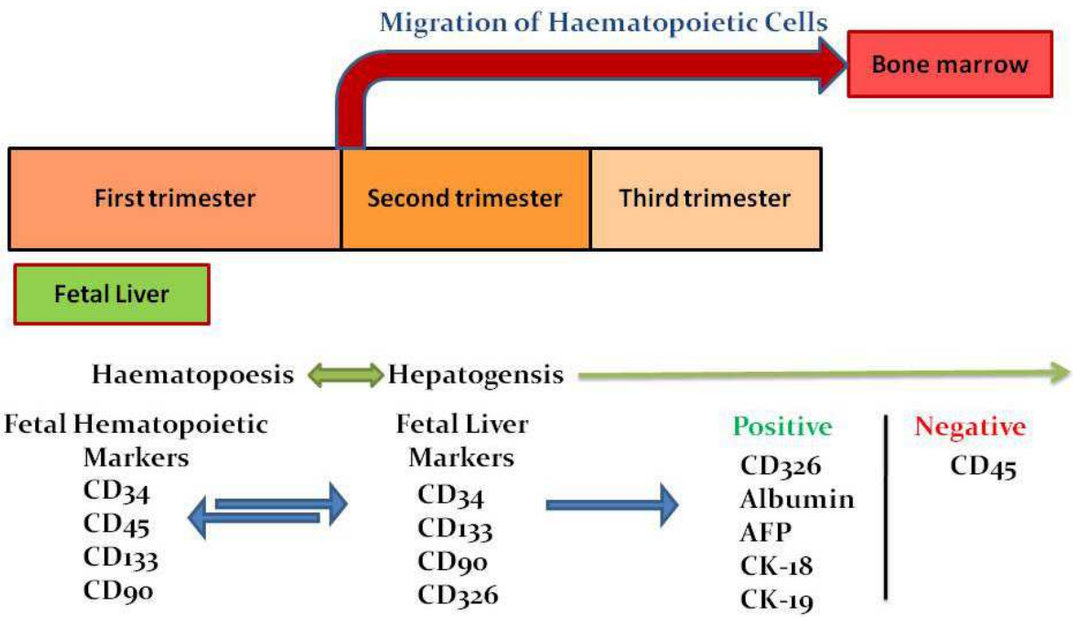

Fig. 1. Transition of cells during liver development

Obtaining good quality and quantity of fetal progenitor cells is very important to ensure repopulation of the liver upon transfusion of these cells. Care must be ensured at every step of tissue collection, isolation of progenitor cells and transfusion of the cells in order to study the efficiency of these cells in treating liver diseases. Some of the important challenges and procedures in these approaches are discussed below.

3.2 Ethical and logistic challenges in obtaining human fetal tissues

The use of human fetal tissues for preclinical or clinical work is always controversial due to religious and ethical issues. The main challenge is the procurement of fetal tissue, therefore the first important step is to identify a maternity/obstetric hospital/department willing to donate aborted fetuses of known gestation period and acceptable medical history for research in good clinical grade condition. The following measures need to be ensured for collection and use of fetal material:

a. **Ethical clearance:** Clearances to work with human fetal material must be obtained from the hospital and the research institute's ethical committee where the fetal cells would be isolated and processed, prior to beginning of the work.
b. **Informed consent:** The mother must be properly informed of the procedure and the written consent must be obtained for the donation prior to the abortion. The process of abortion and donation must be maintained as separate procedures to ensure that the fetus is donated voluntarily and no financial gain is involved in the process.
c. **Screening procedures:** All donors of the fetus must be serologically screened for syphilis, toxoplasmosis, rubella, hepatitis B and C, human immunodeficiency virus 1, cytomegalovirus, parvovirus, and herpes simplex types 1 and 2.
d. **Gestational age:** The gestational age of the fetus is very important and should not cross the second trimester of gestation. The sample should preferably be chosen from an

elective abortion in order to ensure that the tissue is intact and must be collected immediately after the procedure and stored in cold conditions.

e. **Transportation of fetal material:** Once the tissue is collected it must be stored in a clean sterile container with controlled temperature to ensure that the cells are not affected with the temperature changes during the transport. The tissue must be transported to the processing lab as quickly as possible maintaining proper sterile procedures. The cells must be isolated in clean sterile conditions within a maximum of 4 hours of the abortion. If the cells are to be used immediately for transfusion then the processed cells must be enumerated and transported to the hospital in sterile vials ensuring proper temperature conditions.

f. **Storage of cells:** The cells that would not be used immediately should be enumerated and stored with a safe cryo preservative such as DMSO in liquid nitrogen until further use. The viability of the cells during the freeze thaw must be checked before the frozen cells are used for transfusion.

3.3 Preparation of cells from human fetal tissue for therapeutic use:

Once a fetus of the appropriate gestation age is obtained in good medical condition, cell isolation should be done within 4 hours in order to obtain good viability of cells. Two main steps are involved in this process: (a) Isolation of total viable cells from the liver, and (b) enrichment of progenitor cells suitable for transfusion.

3.3.1 Isolation of viable liver cells

The cells in the liver tissue are connected by intercellular connections and tight junctions embedded in an ECM that needs to be disrupted or dissolved for single cell isolation. Three different approaches for isolating viable liver cells with some modifications are principally used:

1. Mechanical dispersion using partial homogenization and forcing the liver tissue through steel meshes [69].
2. The second method of hepatocyte isolation includes removal of calcium and potassium from liver by perfusion and reverse perfusion with calcium binding agent like citrate, Ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), tetra phenyl borate (TBP), pyrophosphate, versine and ATP in calcium free Locke's solution as a sole means of separating intracellular spaces [70, 71].
3. The third method involves the dissolution of intracellular junctions by using proteolytic and matrix dissolving enzymes like trypsin, papain, lysozyme, neuraminidase and pepsin [72, 73].
4. Recently a new protocol for efficient and quick isolation of fetal liver cells from 18-24 weeks of gestation was developed. The protocol involved a 5 step portal vein *in situ* liver perfusion technique using both EGTA and limited exposure to collagenase that resulted in a greater and efficient cell yield [74].

The human fetal liver cells thus isolated can be used as such for treatment of liver failure in model animals and even in experimental clinical trials or can be enriched further for specific progenitor cells.

3.3.2 Enrichment of progenitor cells

The total liver cells that are isolated are a mixture of different population of cells from which the progenitor cells are isolated based on their specific properties by using any of the following enrichment methods. However the method of using the surface markers for enrichment is more efficient in specifically selecting progenitor cells.

a. **Enrichment based on size and density:** Cells of a particular lineage or function have a specific size and mass and this property has been used to separate out desired population of cells from a mixture of cells. Several investigators have used physical methods like density gradient centrifugation and counter flow centrifugation elutriation methods for the enrichment of the hepatic progenitors. Yaswen et al., demonstrated the enrichment of hepatic oval cells (hepatic progenitor) using Centrifugal elutriation (Beckman Instruments, Palo Alto, Calif.) of the cell suspension [75]. Enriched fraction was characterized histochemically for gamma-glutamyl transpeptidase, peroxidase, alkaline phosphatase, glucose-6-phosphatase activities, albumin and alpha-fetoprotein by immunocytochemical methods [75].
b. **Enrichment based on surface markers:** The enrichment of cells can be achieved by using surface markers specifically expressed by the progenitor cells. The process uses antibody specific for the surface marker and separation is achieved by either magnetically tagged (MACS) or fluorescently tagged (FACS) antibodies. In our experience, though FACS is more sensitive for enriching a specific cell type based on the marker expression, the cell yield is not very efficient. Hence enrichment using MACS is more efficient in giving a good yield of the desired population of cells.
   Studies with human livers have shown that Epithelial cell adhesion molecule (EpCAM) is expressed by hepatic stem cells, hepatoblasts and committed progenitors but not expressed in mature hepatocytes [76, 77]. Thus, sorting for EpCAM results in only progenitor cells but in distinct ratios of hepatic stem cells to hepatoblasts depending upon whether the tissue is fetal, neonatal, or adult. In our recent study we have also enriched the hepatic progenitors using EpCAM and further characterized using liver specific and stem cell markers such as CD29, CD90, CD49f, CD34 and we found that EpCAM +ve cells expressed intermediate levels HLA class I but no HLA class II. Our study demonstrated the usefulness of EpCAM as a novel surface marker for enrichment of hepatic progenitors [63]. Earlier we have also used CD34+/CD45- as one the marker for the hepatic progenitors [64].
c. **Separation based on Functional markers:** Certain types of cells express functional receptors on their surface, which transport molecules out of the cells. One such transport protein is the ATP-binding cassette sub-family G member 2 (ABCG2) transporter protein that specifically excludes the Hoechst dye. Separation of progenitor cells can be achieved using this functional marker where the cells specifically exclude the Hoechst dye [78, 79].

3.3.3 Characterization of liver progenitors

The enriched progenitor cells must be further characterized for the expression of hepatic and biliary markers by using methods such as flow cytometry, immune histochemistry and RT-PCR. Hepatic progenitor cells express many markers that are similar to hepatocytes or bile duct cells, and also share some of the haematopoietic markers, AFP, certain keratin

markers [e.g., cytokeratin 19 (CK 19)], and Gamma glutamyl transpeptidase (GGT) [80]. In our study, fetal progenitor cells enriched with EpCAM marker were further characterized for the expression of liver epithelial markers (CK18), biliary specific marker (CK19) and hepatic markers (albumin, AFP) by RT-PCR. FACS indicated that the cells were positive for CD29, CD49f, CD90, CD34, albumin and AFP and negative for HLA class II and CD45. Immunocytochemical staining confirmed the expression of CK18 and albumin [63]. In a study by Lie et al., epithelial progenitor cells from human fetal livers were isolated by cell culture and characterized for their expression of liver epithelial markers (cytokeratin [CK8 and CK18) and biliary-specific markers (CK7 and CK19) by real time PCR. FACS analysis was used to confirm the expression of hepatic markers such as CD117, CD147, CD90, CD44, and absence of hematopoietic markers such as CD34 and CD45. Hepatic differentiation potential of these cells was also confirmed both *in vitro* and *in vivo* [81].

3.4 Treatment of patients with human fetal liver derived cells

Treatment of patients with fetal cells is slowly gaining its importance in the field of cell therapy with encouraging outcomes and improvements in the clinical conditions. However, the success of such procedures is largely dependent on several factors such as age of the patient, type and stage of the disease progression and the route and site of cell injection chosen. Each of these factors are variable for every patient and must be chosen carefully before administering the cells. Some of these factors and the success of the treatment procedures are discussed below:

3.4.1 Categories of patients selected for treatment with fetal cells

Several end stage liver diseases have been treated by liver cell based therapy with different degrees of success; a summary of these findings so far, is given below

1. **Acute liver failure** is characterized by loss of liver cells leading to encephalopathy**.** Initial study by Habibullah et al., showed that fetal hepatocyte transplantation may be beneficial in patients with ALF in grade III or IV encephalopathy [82]. Also, the transplanted hepatocytes may proliferate under the influence of hepatotropic factors thereby increasing their total metabolic and detoxifying capacity. In another study five patients comatose with acute liver failure received transplantation of 1.3×10^9 to 3.9×10^{10} cryopreserved hepatocytes through intrasplenic and intraportal infusion and three patients showed improved result in encephalopathy score and some liver functions [83]. At our centre we have also performed intra-peritoneal transplantation of hepatocytes in a 26 yr old acute fatty liver of a pregnant patient who recovered within two days of transplantation [84].
2. **Metabolic Liver Disease** Hepatocyte transplantation into the liver corrected deficiency. In a landmark trial a child with Crigler-Najjar type I, suffering from dangerous hyper bilirubinaemia, was given $7.5x10^9$ allogenic donor hepatocytes by infusion via portal vein catheter [85]. This procedure resulted in reduction of serum bilirubin levels. Recently, in our study on Crigler-Najjar type I syndrome the patient treated with fetal liver derived hepatic progenitors showed a decrease in the total bilirubin and increase in the conjugated bilirubin [86]. Hepatocyte transplantation in a 4 year old patient with infantile Refsum disease led to partial clearance of abnormal bile acids with pipecholic acid being reduced to 60 per cent of pre-transplantation levels. The child was able to stand and walk 6 months after hepatocyte transplantation [87].

3. **Cirrhosis of liver** This category of liver disease is more appropriate for cell therapy and efficacy of various sources of cells has been demonstrated. Late stage progressive hepatic fibrosis characterized by distortion of hepatic architecture, necrosis of hepatocyte and formation of regenerative nodules leads to cirrhosis. Cell based therapy is emerging as an efficient method of treatment in the management of decompensated liver cirrhosis [88]. Treatment of liver cirrhosis using different sources of cells has been used by several investigators. Procedure and results of these studies has been discussed in section 3.4.3.

3.4.2 Infusion of cells into patients

The survival, proliferation and engraftment of transplanted/infused therapeutic fetal cells are critically dependent on the route of cell delivery and the physiological condition of site of transplantation. The most appropriate site of transplantation is directly into the liver, however because of some limitations of the clinical conditions, several ectopic sites such as the peritoneum, spleen also have been attempted.

3.4.2.1 Sites and routes for cell infusion/transplantation

Several routes to deliver the cells into the liver have been used with varying degrees of success. Cells for transplantation have usually been delivered to liver through hepatic artery [89] or through portal vein [90]. (The different routes for cell infusion are schematically depicted in figure 2).

a. **Hepatic artery**: Hepatic artery can be accessed through trans femoral, trans radial, trans brachial. In our experience hepatic artery route is more convenient compared to hepatic portal vein [91]. Though highly efficient, direct deliveries into liver might pose the risk of occlusion and in certain cases fibrosis, due to portal hypertension and embolism of cells.
b. **Portal Vein**: Portal vein can be accessed by percutaneous trans hepatic approach or trans jugular approach. The hepatic portal vein drains blood from the gastrointestinal tract and spleen into the liver. It is more often used compared to the hepatic artery because multiple vascular accesses are more practical through the vein. However the limitations of being a major procedure and the risk of bleeding must be considered for using the trans-jugular approach for delivery of cells through portal vein
   Accessing portal vein through percutaneous trans-hepatic approach is difficult in the presence of ascites which is a common clinical problem in the end stage of liver disease.
c. **Splenic Approach**: The most appropriate ectopic site is the splenic pulp within the spleen. Spleen has a rich blood supply which is accessible to hepatic portal circulation, leading to the translocation of the transplanted cells to the hepatic sinusoids. Direct intrasplenic injection was suggested as a better method to transplant hepatocytes compared to the splenic artery infusion, since the latter led to vascular occlusion with hepatocytes, gastric erosion, and large areas of splenic necrosis [92]. In a recent study fetal cells were transplanted in a patient with end stage cirrhosis via the intrasplenic infusion through the splenic artery leading to improvement in the clinical condition [74]. Studies have demonstrated that after injection into the splenic pulp, most hepatocytes immediately translocate to splenic veins and then to hepatic sinusoids, although hepatocytes trapped in splenic sinusoids may engraft in the spleen itself [93].

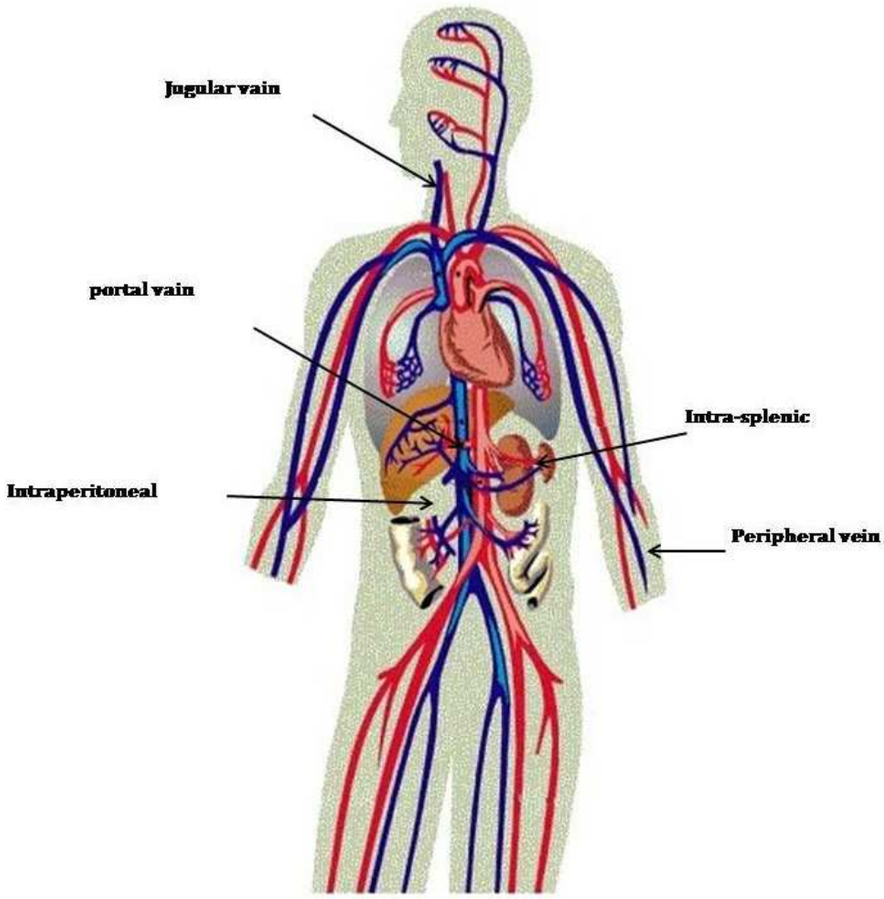

Fig. 2. Different routes for cell infusion

3.4.3 Efficacy and safety evaluation

The safety and efficacy of the fetal and other cell based treatment of end stage liver disease need to be constantly evaluated and revised as per the new protocols as they become available. Comparison of the different cell types and the routes of cell delivery has provided different alternatives for treatment of this disease. For example, our group demonstrated the efficacy of fetal hepatic progenitor cells, delivered through the hepatic artery in 25 cases of de-compensated liver cirrhosis, resulting in significant clinical improvement in more than 80 % cases. The hepatic angiogram showed no sign of thrombosis/narrowing/ischemia in the hepatic artery when analyzed after successive time intervals (Figure 3) [91].

Further data on immune status of allogenic transplantation of human fetal derived progenitors showed no significant changes in the immune status (T cells, NK cells, and Cytokines). The availability of long-term (> 3 years) follow-up data further confirms the safety and efficacy of this therapy. None of the patients recruited in this study developed any procedure/therapy related complications as demonstrated by liver angiograms that were taken pre- and post-transplantation of cells. In another study from our lab [86], infusion of bone marrow derived CD34+ cells in 5 cases by the same route also showed good outcomes in a 6 month follow up.

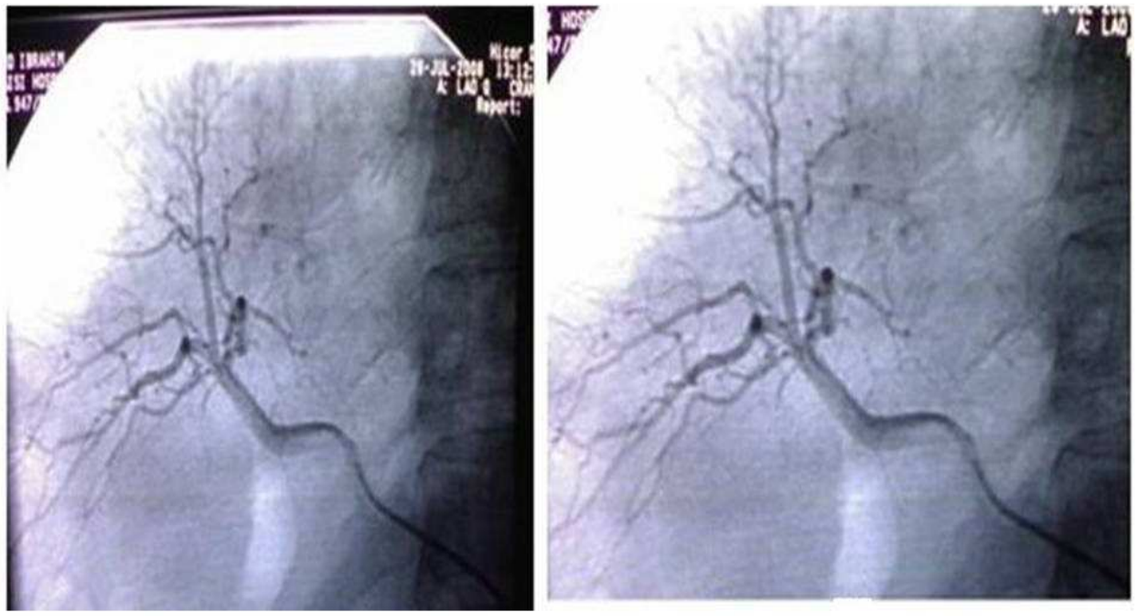

Fig. 3. Comparison of First and second Hepatic arteriogram following administration of Hepatic Progenitor cells to patient with Liver Cirrhosis (Khan et al., 2010)

Several studies have demonstrated the presence of cells of bone marrow origin in the human liver. This was elegantly demonstrated with bone marrow transplantation in female patients who received bone marrow from male donors and were found to be carrying Y-chromosome- and CK8- positive hepatocytes, thus, suggesting that extrahepatic stem cells can engraft in the liver [94]. An improvement of liver function and hepatocyte production was seen in nine patients with liver cirrhosis after the infusion of bone marrow cells through peripheral vein [37]. In another study, 2 patients with alcoholic- induced liver cirrhosis were treated with autologous mobilised HSCs [95]. In a study on 10 patients with chronic end-stage liver disease transplantation of committed progenitor cells and no bone marrow cells via the hepatic artery, showed an improvement in liver function [96]. A significant clinical improvement in the liver function, and Child-Pugh Score was seen in ten patients with advanced liver cirrhosis due to hepatitis B infection following autologous bone marrow infusion [97]. Pai et al., reported a study where autologous expanded mobilised adult bone marrow CD34+ cells were administered via the hepatic artery in 9 patients with alcoholic liver cirrhosis. Significant decrease in serum bilirubin, ALT, and AST levels were observed, whilst the Child-Pugh Scores and radiological ascites improved in 7 and 5 patients, respectively [98].

3.4.4 Tracking and monitoring the fate of transplanted cells

One of the major problems facing the delivery and monitoring of cell transplants is their noninvasive *in vivo* visualization. Tracking of transplanted cells is a very challenging area of cell based therapy. Monitoring transplanted cells becomes imperative in order to understand the route of migration of cells upon injection. It is important that the cells do not enter the pulmonary capillaries as this could lead to complications. In deciding choice of the tracking agent the primary points to be considered are safety of the tracking agent upon administration

and the duration that the tracking agent can provide signals to be able to track the cells. The tracking agent must not cause any effects to the viability or multiplication of the transplanted cells. Though multiple studies have used various biomarkers in pre-clinical studies, their feasibility in human clinical studies warrants further validation.

In one of our clinical studies, tracking of the cells labeled with Tc-HM-PAO was done after cells were infused through hepatic artery (Figure 4). Hepatic scintiography showed that transplanted human fetal hepatic progenitor cells homed in the total lobes of liver with a high rate of engraftment, thereby again reiterating the effectiveness of hepatic artery in the cell delivery. No other clinical complications were observed during and after 6 months of follow-up [91].

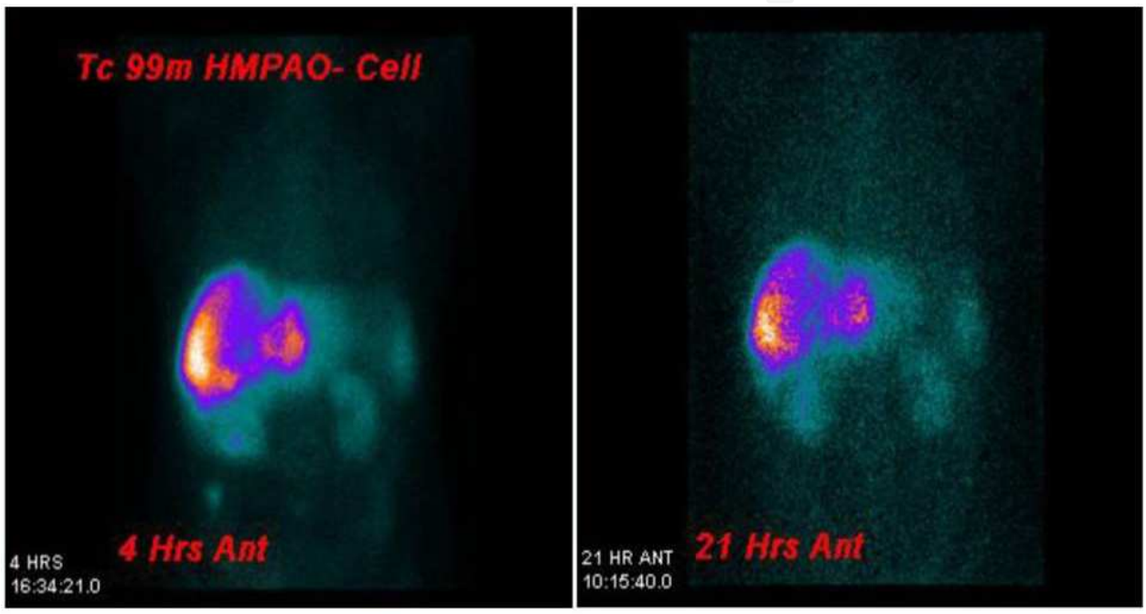

Fig. 4. Tc 99m HMPAO Progenitor cell labeling (Khan et al., 2010)

4. Basic studies in animal models to monitor the progression of the disease and treatment

Over the last decade several advancements have been made in the area of liver disease management. Further research is required to improvise the tissue imaging methods and new technologies are needed to monitor the fate of the cells that are injected. Animal models serve as efficient systems to study these methodologies.

4.1 Non-invasive monitoring of the transplanted cells

Monitoring the fate of transplanted cells over time is important to understand the route of cells migration and also to evaluate the efficacy of the transplanted cells. Several different methods based on magnetic, fluorescence or radio imaging are currently being evaluated for their efficiency in detecting transplanted cells. Though some of the methods are currently being used in clinical settings majority are still in the preclinical stages.

4.1.1 Radionuclide imaging

Radionuclide imaging is a very sensitive method that utilizes radio labeled markers for detection of cells. Single photon emission computed tomography (SPECT) and positron emission tomography (PET), allow the imaging of radio labeled markers and their interaction with biochemical processes in living subjects. SPECT provides the advantages of cell quantification, and lower background signals, but also has disadvantage of a lower spatial resolution compared with MRI and optical imaging [99].

PET is more sensitive than SPECT and permits more accurate quantification of cell numbers. Long term expression of reporter genes such as herpes simplex type 1 thymidine kinase (*HSV1-tk*) for PET imaging is more beneficial than direct and indirect labeling as the cells divide and increase the signal and is also indicative of viability of the cells [100, 101]. The use of reporter gene imaging is currently limited only to animal model studies and needs further validation for its use in clinical settings.

Using radiolabeling the organ bio distribution of human adult hepatocytes and fetal liver cells upon transplantation, was studied in NOD/SCID mice. The cells were labelled with indium-111 (111In)-oxine and technetium-99m (99mTc)-Ultratag or 99mTc-Ceretec and injected via the intrasplenic or intraportal routes. The adult hepatocytes and fetal liver stem/progenitor cells incorporated 111In but not 99mTc labels and the tracking of the cells confirmed that the cells were retained in the liver and spleen without translocating into pulmonary or systemic circulations [93].

4.1.2 Magnetic Resonance Imaging (MRI)

MRI provides a high spatial and temporal resolution in monitoring the distribution, migration, survival and differentiation of the transplanted cells in animals over weeks, *in vivo*. Cells are labeled in advance with contrast agents such as gadolinium-diethylenetriamine penta-acetic acid (Gd-DTPA) for short-term monitoring for upto a week, or superparamagnetic iron oxide (SPIO) for long-term monitoring (reviewed in [102]). Since these contrast agents are exogenous and are often degraded, efforts to develop suitable reporter genes such as the transferrin receptor (TfR) gene that is highly expressed on the target cell membrane have been done for years, however the field is still in its early stage [103, 104]. The major limitation with MRI is the inability to distinguish viable from nonviable cells or proliferating from non-proliferating cell populations. Further it also, cannot distinguish iron-labeled cells from free iron released upon cell death, therefore, iron particle labeling should better be looked as a marker for high-resolution detection of cell location rather than monitoring cell viability in MRI stem cell tracking [105].

4.1.3 Bioluminescence, fluorescence and infra red imaging

Bioluminescence and fluorescence imaging are efficient, noninvasive methods that provide rapid assessments of transgene expression in preclinical models [106]. Bioluminescence imaging is based on transgenic expression of certain bioluminescent genes such as the luciferase gene in transplanted cells and the *in vivo* detection is done by injecting the non-toxic bioluminescent substrate solution and the photons that are generated are captured to provide the images [107-109]. However, bioluminescence is limited by a lower spatial resolution and the inability to produce 3-dimensional and tomographic images [105].

Fluorescent imaging is based on the fluorescence property of a widely used protein such a green fluorescent protein (GFP) that is also introduced into the transplanted cells for stable expression [110]. Due to the low tissue penetrance and non-specific background generated by the autofluorescence of the surrounding tissue GFP reporter cannot be used to reliably track *in vivo* characteristics of transplanted stem cells [109-112].

Imaging in the near-infrared (NIR) wavelength (700–900 nm) spectrum can maximize tissue penetrance in addition to minimizing the autofluorescence from non target tissue. Our lab is currently working on tracking transplanted cells using the fluorescent dye Di-D. EpCAM +ve human fetal liver cells were labeled with Di-D and injected in the liver of nude mice to monitor their survival and engraftment of the liver. Using Di-D the labeled cells were imaged with KODAK Fx PRO animal imaging system at various time points indicating the persistence of fluorescencent labeled EpCAM+ve cells upto 110 days post transplantation (Figure 5). The fluorescence images of animal were overlaid with the X-ray images of the animal. Further studies on the safety and efficacy of the dye needs to be confirmed before clinical trials could be attempted.

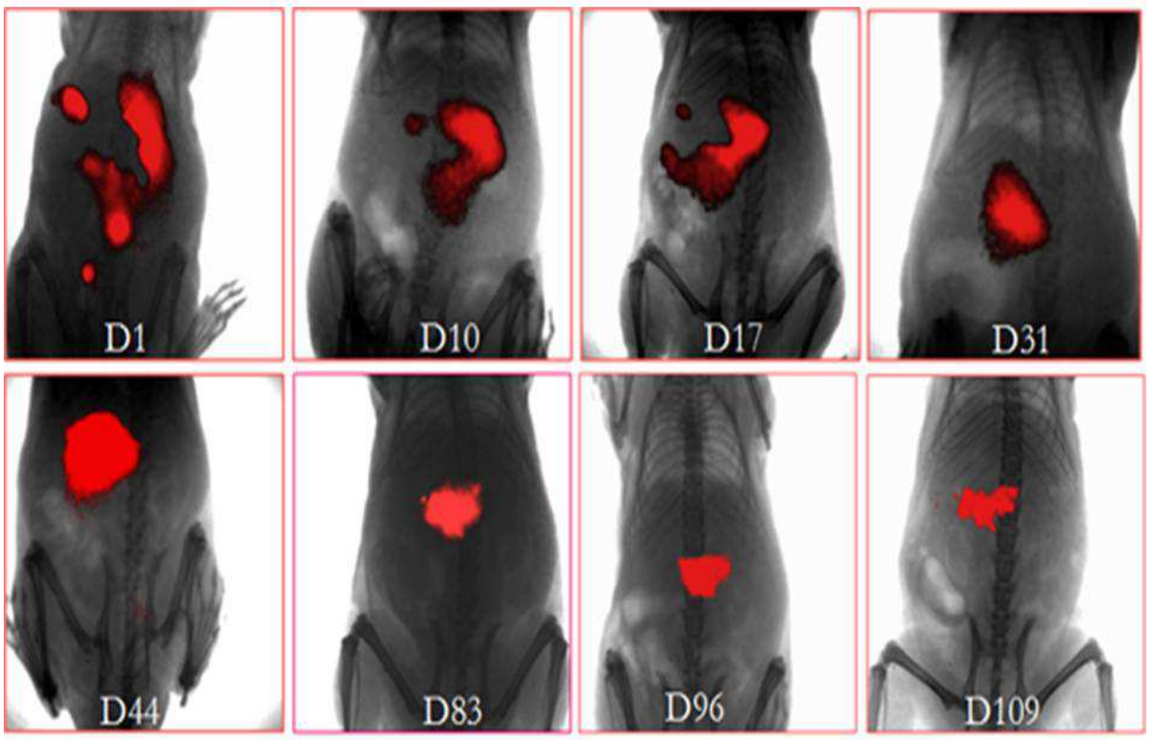

Fig. 5. *In vivo* images of nude mice after intra hepatic transplantation with DiD labeled EpCAM +ve cells at different time points (Unpublished data)

4.2 Need for more non- invasive tissue imaging methods to monitor disease progression

Non-invasive tissue imaging methods to detect fibrosis play an important role in the management of liver disease. Methods to monitor the disease progression are as important as the treatment procedures, as timely detection of fibrosis could help in prevention of the

further progression and restore the liver functions with minimal intervention or cell therapy. Imaging methods offer an invaluable diagnostic tool in detection and monitoring the disease progression.

The conventional methods of imaging such as the ultrasonography and computed tomography are routinely used in the evaluation of fibrosis but with limited success in predicting the stage of the disease. The more recent methodologies based on elastography such as the fibroscan (ultrasound elastography) and Magnetic Resonace elastography (based on magnetic resonance) measure the stiffness of the tissue and correlate to the extent of disease progression and are more sensitive to detect fibrosis. However, the sensitivity of these methods are limited to detect fibrosis only in later stage of disease progression and new sensitive methods need to be developed that can predict the early fibrotic changes of the liver. Since fibrosis is characterized by extensive and unorganized accumulation of ECM with the principal component being collagen, our lab has initiated work on this front by targeting collagen to detect fibrosis. We propose to achieve this by using probes that are specific to collagen and combine these to a fluorescent or magnetic reporter molecule that would be detected by imaging methods. The amount of collagen detected would be correlated to the stage of fibrosis. Studies are currently being carried on rat and mouse models and would need extensive confirmation before these can be extended to human studies.

5. Conclusions

Cell based treatment is a promising area which is slowly gaining importance as a therapeutic approach for liver diseases. Preclinical and clinical trials with different types of adult cells have provided evidence for their usefulness in treating cirrhotic liver disease though with certain limitations. Recently, human fetal progenitor cells with their low immunogenicity and a good proliferative capacity are emerging as safe and potential sources for treating liver diseases. However, the efficiency of these cells in treating various types of liver diseases is yet to be established. Long term monitoring of the fate of the transfused cells also remains a challenging area that needs to be addressed before these cells can be used as routine treatment options for liver diseases. Improvised methods of cell isolation and infusion in patients and the development of faster and efficient non-invasive methodologies for detecting fibrosis at early stages would be an important step towards the management of liver diseases.

6. Acknowledgement

The work reported in this review was supported by Grants No. GAP220, from the Department of Science and Technology, New Delhi to GP and GAP338 from Indian Council of Medical Research New Delhi to GP and AAK.

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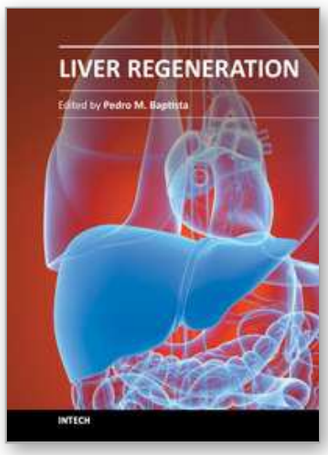

Liver Regeneration

Edited by PhD. Pedro Baptista

ISBN 978-953-51-0622-7
Hard cover, 252 pages
Publisher InTech
Published online 16, May, 2012
Published in print edition May, 2012

Doctors and scientists have been aware of the "phenomenom" of liver regeneration since the time of the ancient Greeks, illustrated by the mythic tale of Prometheus' punishment. Nevertheless, true insight into its intricate mechanisms have only become available in the 20th century. Since then, the pathways and mechanisms involved in restoring the liver to its normal function after injury have been resolutely described and characterized, from the hepatic stem/progenitor cell activation and expansion to the more systemic mechanisms involving other tissues and organs like bone-marrow progenitor cell mobilization. This book describes some of the complex mechanisms involved in liver regeneration and provides examples of the most up-to-date strategies used to induce liver regeneration, both in the clinic and in the laboratory. The information presented will hopefully benefit not only professionals in the liver field, but also people in other areas of science (pharmacology, toxicology, etc) that wish to expand their knowledge of the fundamental biology that orchestrates liver injury and regeneration.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Chaturvedula Tripura, Aleem Khan and Gopal Pande (2012). Cell Based Therapy for Chronic Liver Disease: Role of Fetal Liver Cells in Restoration of the Liver Cell Functions, Liver Regeneration, PhD. Pedro Baptista (Ed.), ISBN: 978-953-51-0622-7, InTech, Available from: http://www.intechopen.com/books/liver-regeneration/cell-based-therapy-for-chronic-liver-disease-role-of-fetal-liver-cells-in-restoration-of-the-liver-c

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