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Cell Transplantation – A Possible Alternative to Orthotopic Liver Transplant (OLT)

Kristen J. Skvorak et al.*
University of Pittsburgh
USA

1. Introduction

The progress made in the field of liver organ transplantation has revolutionized the treatment of a wide spectrum of liver diseases. Orthotopic liver transplantation (OLT), which requires removal of the entire native liver and transplantation of a high quality graft, has become an almost routine procedure with 1-year survival rates higher than 80%. However, with the ensuing interminable increase in the waiting list, the current major limitation is the considerable shortage in organ donors and the need of timely availability of suitable livers. As a result, although death rate after surgery is slowly decreasing, the number of total deaths in waiting list patients is steadily rising. Several solutions have been proposed to overcome this problem, such as legislative measures, mass media campaigns, optimization of available organ allocation, or innovative surgical techniques such as split-liver, living donor, non-heart beating donor, and domino transplantation. However, these measures have been met with only limited success in providing enough liver grafts (Neuberger, 2000; Thalheimer & Capra, 2002). Hence, the research community endeavoured to establish clinical alternatives to liver transplantation. Cell-based therapies are emerging as an alternative to whole-organ transplantation, which has shown initial promise in both animal models and clinical cases. This novel technique may provide functional liver support while the native liver regenerates in patients of acute liver failure, may provide a short-term “bridge” to sustain critical patients until OLT, or may aid in replacing a missing enzyme function in metabolic conditions with the aim of avoiding OLT. Some of the most promising cells types that could be used in this emerging field are hepatocytes, embryonic stem cells (ESC), mesenchymal stromal cells (MSC), amnion epithelial (AE) cells, and induced pluripotent stem cells (iPSC).

2. Cell transplant versus OLT

Although still in the experimental stages, cell transplant (CTx) has a variety of potential advantages over whole liver transplant. OLT requires major invasive surgery associated with long recovery times and a high prevalence of post-surgical complications such as infections, renal failure, and acute rejection, which could all contribute to patient mortality. The financial cost of OLT and subsequent lifelong immunosuppression therapy is substantial,

* Roberto Gramignoli, Marc C. Hansel, Suleyman Uraz, Veysel Tahan, Kenneth Dorko, Fabio Marongiu and Stephen C. Strom

and long term immunosuppression has also been linked to an increased incidence in cancers. Finally, the number of livers needed for transplant greatly outnumbers available livers, and timing is critical. In contrast, CTx is less invasive, less expensive, and is associated with less severe and fewer complications as well as shorter recovery times. Theoretically, stable patients, such as those with a metabolic disease, could potentially be given an infusion of cells as an outpatient procedure. Cells for transplant can be banked and cryopreserved for almost instant availability; therefore, procedural timing would no longer be a major concern. One significant benefit of CTx is that patients would retain their native liver. This is of particular relevance for patients with metabolic liver diseases. For example, a patient with Maple Syrup Urine Disease (MSUD) has a mutation in the enzyme complex that catalyzes the permanent degradation of branched-chain amino acids (BCAA), but can perform all other necessary liver functions. Therefore, transplanted cells would not need to provide complete liver support. In addition, since a metabolic disease patient is not reliant on the donor cells for other liver functions, loss of a graft or failure of the cells to perform would only return the recipient to pretransplant conditions. Cells can also be infused into patients multiple times, and OLT remains an option if CTx proves insufficient. Less immunosuppression may also be required, though this would likely depend on the type of cells used, the number of cells infused, and each patient's individual needs.

3. Cell types, utility for transplant, and major benefits / concerns

Cell transplant may be clinically useful for cell support for acute liver failure, as a “bridge” therapy to whole liver transplantation, or for the treatment of metabolic liver disease (Strom & Ellis, 2011). Hepatocytes, as well as many stem or stem-like cells (ESC, MSC, AE, and iPSC), are all being investigated for use in this novel yet promising branch of regenerative medicine. Each cell type has its own associated risks and benefits, which will be discussed separately.

3.1 Hepatocytes

The adult human liver consists of approximately 250 billion hepatocytes organized in about one million hepatic lobules. Each capillary leads to a lobule. Hepatocytes, the basic metabolic cell of the liver, constitute approximately 65–80% of the cell population of the liver. These cells are involved in protein synthesis, storage and transformation of carbohydrates, synthesis of cholesterol, bile salts and phospholipids, detoxification, and modification and excretion of exogenous and endogenous substances (Kaplowitz, 1992). The hepatocyte also initiates the formation and secretion of bile. Other important cells of the liver include Kupffer cells, stellate cells, and endothelial cells. Kupffer cells are specialized macrophages located in the liver that form part of the reticuloendothelial system. Their development begins in the bone marrow with the genesis of peripheral blood monocytes, and completing their differentiation into Kupffer cells in the liver. The primary functions of Kupffer cells are to recycle old and non-functional red blood cells, phagocytosis, and clearance of pathogens. Stellate cells, also known as Ito cells, are pericytes found in the perisinusoidal space (space of Disse) and represent ~5-8% of the total liver cell number. In normal liver, stellate cells are largely quiescent, store vitamin A, and have cell body protrusions that wrap around sinusoids. The stellate cell is the major cell type involved in liver fibrosis, which is the formation of scar tissue in response to liver damage. When liver is damaged, stellate cells can change into an activated state, and are responsible for secreting

collagen and other extra-cellular molecules which can lead to cirrhosis. Endothelial cells constitute the wall of the liver sinusoids. They lie on a discontinuous extracellular matrix, creating a narrow extravascular fluid compartment into which hepatocytes project microvilli. This arrangement maximizes the surface area of hepatocytes with the extravascular fluid space and permits free movement of solutes into contact with the hepatocyte plasma membrane.

In healthy individuals, regeneration is slow; it is commonly accepted that liver is replaced by normal tissue renewal approximately once a year. The liver is largely quiescent with only 1:1000 hepatocytes in mitosis at any given time. This number decreases with increasing age, making regeneration slower and less complete in older animals (Steer, 1995). However, this slow cellular turnover is quickly altered when a chemical or physical trauma occurs to cause a significant loss to the liver. Sudden and massive hepatocyte proliferation then occurs due to a rapid increase in mitotic division resulting in restoration of functional liver mass (Bucher, 1963).

3.1.2 Hepatocyte transplantation and route of administration

Hepatocytes are capable of rapid proliferation as well as complete and functional regeneration of the liver following injury. Thus, this capacity for self-renewal has led some to regard the hepatocyte as essentially a “unipotent” stem cell. As early as 1977, hepatocyte transplantation (HTx) has been recognized as an attractive option for the management of metabolic liver disease (Groth et al., 1977). Groth and colleagues demonstrated that intraportal HTx in glucuronosyltransferase-deficient rats improved hyperbilirubinemia. Since then, continuing preclinical research determined that HTx can support liver function and improve survival in animal models of acute liver failure suggesting it had potential clinical application (Gupta & Chowdhury, 2002). There have also been a large number of studies with various animal models that show the efficacy of hepatocyte transplantation to correct metabolic liver disease (as reviewed in Malhi & Gupta, 2001; Strom et al, 2006). Importantly, Harding’s group showed the correction of murine phenylketonuria (PKU) despite low engraftment of cells (Hamman et al., 2005); Harding & Gibson (2010) later suggest only 10-20% repopulation may be sufficient to correct PKU clinically. Our group also recently reported a significant partial correction of murine intermediate MSUD despite very low (~3%) repopulation of the liver (Skvorak et al., 2009a; 2009b). As a result, HTx has gained attention as a potential therapeutic intervention for a number of liver diseases, and transplantation of hepatocytes corresponding to 1-5% of total liver mass (1.5-9.0 billion hepatocytes) can be expected to have a positive impact. It has been determined that approximately 3.5-7.5% of liver mass can safely be transplanted in one transplant event (Fox et al., 1998), whereby the transplant may be divided in up to 6 separate infusions over a number of hours. CTx is generally associated with an increase in portal pressures as blood flow is restricted by plugs of donor hepatocytes (Gupta et al., 1999). However, if transplanted cells are in the range of 5% of the total liver mass, this increased portal pressure usually resolves within minutes or hours. When portal pressures return to normal, or at least decrease to acceptable levels, it is then safe to infuse more cells. At present, the majority of hepatocyte transplant procedures have been performed in adults with acute or chronic liver failure, though HTx as a therapeutic alternative to treat metabolic hepatic disease is becoming more accepted. Thus far, there have been four reported clinical cases of

recovery from acute liver failure following HTx (Table 1), though the use of cell transplant as a “bridging” therapy to OLT is more common in both acute and chronic liver failure. Most published articles report a positive impact of HTx in clinical studies, and results are in general agreement with preclinical data using animal models. Table 1 summarizes clinical hepatocyte transplant studies to date, as well as significant results of those studies.

HTx as an alternative treatment for metabolic liver disease is an appealing proposal. The progression of inherited metabolic liver disease usually varies less than cases of liver failure. In addition, objective parameters such as laboratory data (i.e. bile acid, clotting factors, etc.) can be determined to unequivocally assess the efficacy of the treatment. On the other hand, the situation is rarely immediately life threatening and often acceptable conventional therapies are available, such as a special diet. Therefore, the potential benefit must be carefully weighed against any possible complications, such as immunosuppression, embolisation of the pulmonary vascular system, sepsis, or hemodynamic instability. HTx is more often done as therapy for inborn errors of hepatic metabolism in which a specific absent protein can be measured from transplanted unmodified donor hepatocytes expressing the gene. The use of hepatocyte infusions to correct inborn errors of metabolism is logical when a specific metabolic deficiency, with well-studied animal modelling, can be measured. Then, after infusion of donor liver cells natively expressing the required gene, objective measures of required hepatocyte mass, engraftment percent, and survival advantage can be obtained. Thus far, therapeutic benefit has been seen clinically in the treatment of disorders of the urea cycle (citrullinemia, OTC, argininosuccinase lyase deficiency), familial cholesterolemia, Crigler-Najjar, biliary atresia, infantile refsum disease, Factor VII, and Glycogen storage disease type 1a & 1b (summarized in Table 1). To avoid the need for immunosuppression or the risk of rejection, transplantation of genetically modified autologous hepatocytes may also be an option, such as in a clinical study to treat familial cholesterolemia (Grossman et al., 1991). In this study, retrovirus was used to transduce and correct a patient’s deficient hepatocytes, which were then infused back into the patient to yield a partial correction of the disease. HTx to treat progressive familiar intrahepatic cholestasis and A1AT were also attempted, but no clinical benefit was determined likely due to the presence of fibrosis in the native liver (Strom et al., 1997a; Strom et al., 1999; Hughes et al., 2005). With respect to long-term engraftment, it will be important whether the transplanted hepatocytes will gain a selection advantage over the recipient’s cells. Damage or injury to the native liver triggers rapid proliferation of healthy hepatocytes; theoretically this would provide transplanted hepatocytes a selected growth advantage over native cells in patients with acute or chronic liver failure. Importantly, the livers of metabolic disease patients are not injured or damaged in most cases; transplanted cells would likely not receive selection advantage over the recipient’s cells. Therefore, higher numbers of transplanted hepatocytes, a need for better cell engraftment, and repeated transplantations may be necessary for the successful treatment of metabolic liver diseases.

Cell therapy of end-stage liver disease (i.e. cirrhotic livers) is more problematic. Infusion into the liver via the portal vein is the preferred method of transplant in cases where liver architecture is intact (i.e., metabolic diseases, or in the case of acute liver failure). It is known from animal studies that hepatocytes infused via the portal vein disperse with the portal blood flow and finally translocate to the hepatic sinusoids in the periportal region of the liver lobules (Sokal et al., 2003). Single cells succeed in traversing the endothelial barrier and integrate into the parenchyma. After re-establishing intercellular contacts with neighbouring

Liver Disease	Outcome	References
α 1-antitrypsin (A1AT)	No clinical benefit likely due to the presence of fibrosis	(Strom et al., 1997a; Strom et al., 1999)
Acute liver failure	Reversal of disease	(Fisher et al., 2000; Soriano, 2002; Fisher & Strom, 2006; Ott et al., 2006)
Argininosuccinate lyase deficiency	Complete correction	(Stephenne et al., 2006)
Biliary atresia	Partial correction - slow and continuous decrease in bilirubin levels	(Khan et al., 2008)
Chronic liver failure	Bridge to OLT	(Bilir et al. 200; Strom et al. 1997b; Fisher & Strom, 2006; Strom et al., 1999)
Citrullinemia	Partial correction - decreased citrulline and circulating ammonia at 6 months post- cell infusion	(Meyburg et al., 2009a)
Crigler-Najjar type 1	Partial correction - slow and continuous decrease in bilirubin levels; evidence of long term correction by hepatocyte graft (one patient was followed for > 1.5 years)	(Fox et al., 1998; Dhawan et al., 2004; Ambrosino et al., 2005)
Familial cholesterolemia	Partial correction - cholesterol decrease and transgenic expression >4 months	(Grossman et al., 1991) *
Glycogen storage disease type 1a & 1b	Partial correction - patients could maintain blood glucose between meals as well as higher and sustained glucose levels at meals	(Muraca et al., 2002; Lee et al., 2007)
Infantile refsum disease	Partial correction - improved fatty acid metabolism, reduced pipecolic acid and bile salt levels, improved strength and weight gain	(Sokal et al., 2003)
Inherited Factor VII deficiency	Partial correction - reduced FVII requirement 80%	(Dhawan et al., 2004)
Ornithine transcarbamylase deficiency (OTC)	Partial correction - ammonia and glutamine levels were normalized following transplant. Most required OLT at a later date.	(Strom et al., 1997a; Horslen et al., 2003; Mitry et al., 2004; Stephenne et al., 2005; Puppi et al., 2008; Meyburg et al., 2009a; 2009b)
Progressive familial intrahepatic cholestasis	No clinical benefit likely due to the presence of fibrosis	(Hughes et al., 2005)

* use of genetically modified autologous hepatocytes

Table 1. Summary of clinical HTx to treat chronic liver failure, acute liver failure, and inherited metabolic diseases

host cells, transplanted hepatocytes may start to proliferate when sufficient space is made for the infused cells. Donor cells and their descendents form gradually increasing clusters, thus finally repopulating the recipient liver. However, cirrhotic livers contain abnormalities of the hepatic architecture, as well as loss of functional hepatocytes, which contributes to the decrease in liver function. In addition, intrahepatic portal venous shunts may prevent an efficient exchange between hepatocytes and blood plasma, and cell infusions may cause prolonged portal hypertension and embolization in the lung (Strom et al., 1999). Preclinical HTx studies conducted on rat cirrhosis models discovered significantly increased intrapulmonary translocation of donor cells due to portal shunting (Gupta et al., 1993). Due to cirrhotic changes in the liver and associated portal hypertension, the infusion of donor hepatocytes into the liver via the portal vein without first restoring the normal liver architecture would likely cause serious complications in patients with portal hypertension. For safety purposes, transplantation into the spleen is preferable (Strom et al., 1997b; Fisher & Strom, 2006). Direct intrasplenic injection produced engraftment that was far superior to that obtained using splenic artery infusion and resulted in fewer serious complications (Nagata et al.; 2003). However, it is still unknown whether the human spleen is capable of accommodating a sufficient number of functional hepatocytes to compensate for the cirrhotic liver. For example, alcoholic cirrhotic patients showed only transient clinical improvement after treatment by splenic HTx (Strom et al., 1999; Sterling & Fisher, 2001). Another strategy that has been getting recent attention is the use of a bioartificial liver (BAL) to support metabolic function and regeneration (Koenig et al., 2005; Carpentier et al., 2009). Several devices are being tested clinically (as reviewed in Carpentier et al., 2009). However, current limitations of BAL devices are cost and, due to the shortage of allogenic hepatocytes, their prevalent use of porcine hepatocytes, which carry the risk of infection with porcine endogenous retrovirus and anaphylaxis (Chamuleau et al., 2005). Treatment of cirrhotic livers by CTx requires extra consideration regarding transplant site, cell number, and overall safety of the procedure. The continuing development of BAL is promising, but there are more challenges to overcome in this area before these devices can be considered a cost-effective and safe option for the treatment of liver disease.

3.1.3 Hepatocyte isolation, culture, and cryopreservation

The major source of hepatocytes for HTx are livers that were rejected for OLT. Some of the most common reasons that procured livers are not used for transplantation are as follows: unavailability of a matched recipient, physical damage to the liver, pre-existing liver diseases, breach of sterility during the procurement process, high liver fat content (steatosis), inappropriate age (too old), or inappropriate warm ischemic time or cold storage time (cold ischemia). Though these organs may not be therapeutically useful for OLT, viable cells for CTx may still be acquired.

Hepatocyte isolation was first employed in the late-1960s for rat livers (Howard et al., 1967; Berry & Friend, 1969). In 1976, the traditional two-step collagenase perfusion technique was developed for rat tissue (Seglan, 1976), which was later adapted for use with human tissue (Bojar et al., 1976). Another widely used method, which yields a high number of viable cells per gram of whole liver tissue, is the three-step collagenase perfusion technique (Dorko et al., 1994; Nakazawa et al., 2002; Mitry et al., 2004; Alexandrova et al.; 2005). More recently, the increasing application of these approaches in clinical grade cell therapies require the standardization of cell isolation procedures in accordance with GMP conditions (Gramignoli

et al., 2011). In general, after collagenase has disassociated the hepatocytes from the connective tissue, cells are separated by low speed centrifugation, and the hepatocyte pellets are washed with ice-cold buffer solution to purify the cells. The number and quality of the isolated hepatocytes vary depending on the composition of perfusion buffer solutions, the type and concentration of enzyme, and the type and quality of the tissue source used. Further purification of viable cells can be obtained through the use of the Percoll centrifugation technique (Olinga et al., 2000), though extensive loss in cell number (20-40% cell recovery) is a major disadvantage of this method (Dorko et al., 1994). Cell viability is determined using trypan blue exclusion. However, *in vitro* viability may not reflect good cell function *in vivo*.

The primary requirement for both short-term and long-term culture of hepatocytes is their ability to efficiently attach to the culture plate. The culture dish should be pre-coated with a suitable attachment factor such as collagen (type I or IV) or Matrigel, which contains a mixture of extracellular matrices (Blaauboer & Paine, 1979; Chen et al., 1998). However, even under currently optimal *in vitro* cell culture conditions, mature human hepatocytes typically do not survive or maintain mature functionality for periods longer than 1-2 weeks, proliferation is extremely poor, and they appear to de-differentiate and lose hepatic potential (Tanaka et al., 2006, Nahmias et al., 2007).

A shortage of donor liver tissue for the isolation of human hepatocytes necessitates the development of improved cryopreservation techniques for long-term storage. There are several reports describing various cryopreservation techniques and some of the associated complexities of the procedure (Diener et al., 1993; Terry et al., 2005; 2007). Hepatocytes are typically cryopreserved in suspension, which can occur immediately following isolation. No culture step is needed. The ultimate goal of any improved cryopreservation protocol is to minimize sudden intracellular formation of ice crystals that could result in ultrastructural damage, and thus maintain cell viability, attachment, and metabolic activity upon thawing. Storage time of cryopreserved hepatocytes at temperatures well below -100°C (e.g. liquid nitrogen or -140°C freezers) may play an important role in the quality of thawed cells. Cells are resuspended in ice-cold media (usually Belzer solution, also known as UW) containing cryoprotective agents. UW has been well established in the literature to have the best results in terms of viability and recovery. The cryoprotectants used can be permeating (e.g., DiMethylSulfoxide (DMSO), glycerol) or non-permeating molecules (e.g., polymers, sugars). DMSO is the cryoprotectant of choice because it is permeating and highly soluble. It is able to enter cells and reduce injury through reduction of ice crystal formation during freezing. DMSO, being a polar solvent, may also stabilize the plasma membrane by electrostatic interactions. The concentration of these cryoprotectants, as well as the rate at which they are added, final cell density, and freezing rate may also be crucial factors contributing to viability upon thawing. The standard optimum thawing protocol for hepatocytes is rapid thawing at 37°C with slow dilution of the cryoprotectant (to reduce osmotic imbalances) at 4°C (to reduce possible toxicity of the cryoprotectant) (Karlsson et al., 1993; 1996; Pegg, 2002). Upon thawing, cells are then washed to remove cryoprotectant to avoid potential adverse affects in patients.

3.1.4 Benefits / concerns

Due to the undeniable success of OLT, it is reasonable to use all suitable donor livers for organ transplantation. Therefore, an advantage of HTx is that it would not require obtaining

livers that could be used for OLT, which would only further stress an already stressed system. HTx would be using liver tissue that would otherwise be discarded. In addition, multiple patients could be treated with hepatocytes from a single tissue donor, and potentially, in cases of metabolic disease, a patient's autologous hepatocytes could be collected, genetically manipulated to correct the deficiency, and infused back into the patient. Nonetheless, there are still many problems associated with the use of hepatocytes. Despite the use of discarded tissue, the current major limitation is the availability of human hepatocytes. Although hepatocytes *in vivo* have remarkable proliferation potential, primary hepatocytes proliferate very poorly *in vitro*, appear to de-differentiate and lose their hepatic potential, and display very limited survival (Tanaka et al., 2006, Nahmias et al., 2007). Therefore, the collection of hepatocytes for HTx is still limited by the availability of fresh liver tissue as cells cannot be expanded in culture. The numbers and/or quality of hepatocytes isolated from non-transplantable livers will not allow a widespread application of HTx. A second major limitation is the need for timely availability of hepatocytes. If hepatocytes cannot be successfully cryopreserved and thawed, some advantages of CTx over OLT are lost. Successful cryopreservation is needed for establishment of cell banks, which would allow cryopreserved hepatocytes to be available for emergency use in acute and chronic liver diseases, or for planned or repeated use in patients with liver-based metabolic disorders. A third major limitation is the consistently poor quality of cells after cryopreservation. Hepatocytes are very sensitive to freezing damage, and three distinct modes of cell death have been identified: cell rupture by the formation of ice crystals, necrosis, and apoptosis (Baust, 2002). Loss of membrane integrity, and thus leakage of important enzymes and cofactors which affect liver function, low attachment efficiency, and a loss in viability of 50% or greater is typical. This situation will remain unaltered until alternatives to primary hepatocytes becomes available, which are discussed in the next sections of this chapter, or more efficient methods of cryopreservation and storage of hepatocytes, as well as cell recovery from cryopreservation are determined.

3.2 Embryonic stem cells

Embryonic stem cells (ESC) are derived from totipotent cells of the inner cell mass of the blastocyst, an early stage of the developing embryo (Thomson et al., 1998). ESCs are pluripotent, meaning they can differentiate into all three germ layers (ectoderm, mesoderm, and endoderm), and express many specific gene factors that have come to be known as cell markers of pluripotency. Common markers of "stemness" include stage specific embryonic antigens (SSEA) 3 & 4, and the tumor rejection antigens (TRA) 1-60 & 1-81 (Thomson et al., 1998), while some common molecular markers include OCT-4, SOX-2, and Nanog, as well as high expression of telomerase reverse transcriptase (TERT) (Thomson et al., 1998; Chambers et al., 2003). Telomerase maintains telomere length and adds telomere repeats to chromosome ends, which is important in a cell's replicative lifespan (Vaziri & Benchimol, 1998). However, high levels of telomerase activity are also found in 80-90% of human tumor samples (Chen & Chen, 2011). ESCs will readily become tumorigenic *in vivo* when injected into severe combined immunodeficient (SCID) mice forming either teratomas, tumors comprised of cells from all three germ layers, or teratocarcinomas, which are more aggressive, malignant teratomas (Ben-David & Benvenisty, 2011). In fact, teratoma formation is so characteristic of ESCs, it has become one of the most informative tests of pluripotency for ESC-like cells, such as induced pluripotent stem cells (iPSC). ESCs also display genetic instability (i.e. aneuploidy) *in vitro*, another unfortunate characteristic they

share with cancer cells (Spits et al., 2008). Furthermore, ESCs express very low human leukocyte antigen (HLA) class I antigens (Baroja et al., 2004), and almost undetectable levels of HLA class II antigens and co-stimulatory factors (Drukker et al., 2006). ESCs are still subject to immune system targeting, however. Low expression of HLA class I molecules is sufficient to induce acute rejection through the action of cytotoxic T-cells and affect treatment tolerance (Robertson et al., 2007; Drukker et al., 2006), which suggests that immunosuppression would still be required if patients received stem cell-derived CTx.

Sustaining pluripotency *in vitro* requires continued expression of Nanog and OCT-4 (Chambers et al., 2003). The expression of these factors are maintained through co-culture with a feeder cell layer, most commonly mouse embryonic fibroblasts (MEFs), and either the addition of basic fibroblast growth factor (bFGF) for human ESC, or leukemia inhibitory factor (LIF) for mouse ESC. Without optimal culture conditions, ESC will rapidly and spontaneously differentiate into cells from all three germ layers (Chambers et al., 2003). Finally, ESCs are indefinitely self-renewing theoretically providing an unlimited therapeutic source of cells for regenerative medicine (Thomson et al., 1998).

3.2.1 Inducing hepatic differentiation in ESC and laboratory / clinical data

ESCs will spontaneously differentiate simply by removing factors and/or allowing the formation of spheroid clumps known as embryoid bodies (EB) in culture. ESCs can also be made to differentiate along a defined lineage through exposure to specific growth factors. In a developing embryo, signals from the cardiac mesoderm and septum transversum mesenchyme specify endoderm to accept a hepatic fate. It was eventually determined that FGFs and bone morphogenic proteins (BMPs) can mimic the appropriate signals and thus induce endoderm towards a hepatic fate (Jung et al., 1999). From there, targets of BMPs and FGFs, as well as other *in vivo* hepatic regulatory genes, such as FoxA genes and the GATA and hepatocyte nuclear factor (HNF) transcription factors, defined additional molecules tested in differentiation studies to produce hepatocyte-like cells from ESC. Currently there are many published protocols to differentiate ESC into various cell types from all three germ layers, which have been reviewed elsewhere (Trounson, 2006; Zaret & Grompe, 2008; Soto-Gutierrez et al., 2008; Sancho-Bru et al., 2009). Hepatocyte-like cells that express α -fetoprotein (AFP), albumin, cytochrome P450 (CYP450), cytokeratin (CK) 18, and display epithelial-like morphology have all been extensively described. However, expression of these few factors does not guarantee the differentiated stem cell is a “hepatocyte”; hepatocyte-like stem cells may express a few hepatic genes, but they could also be negative for many others important to hepatic function (Soto-Gutierrez et al., 2008). In addition, some of these hepatic markers are not limited to hepatocyte expression, such as CYP450. Therefore, there must be a more stringent check list to determine when a stem cell is considered to be completely differentiated.

There have been many articles describing ESC derived hepatocyte-like cells transplanted into liver-damaged mice (e.g. review by Banas et al. 2007) but few have determined the cells significantly contribute to improved liver function and regeneration. Induction rates remain low regardless of the method used, and general hepatic function of the cells, even once transplanted, were very limited when compared to mature hepatocytes (Sharma et al., 2008). However, a successful report described ESCs demonstrating liver function able to overcome liver damage in mice (Heo et al., 2006). This is encouraging for the field of liver disease, but

more efficient differentiation and transplantation techniques must be established. Clinical ESC therapy for liver disease is not currently realistic, but at present there are four ongoing ESC clinical trials targeting other organs in the United States (Trounson et al., 2011). Two trials are in Phase I and are targeting spinal cord injuries or spinal muscular atrophy, while the remaining two are in Phase I/II and are targeting Macular Degeneration. All trials involve ESCs that were first differentiated in culture prior to transplantation.

3.3 Mesenchymal stromal cells

Mesenchymal stromal cells (MSC), formerly known as mesenchymal stem cells, are multipotent non-hematopoietic adult stem cells that have been isolated from a variety of tissues, including bone marrow, adipose tissue, Wharton's jelly, umbilical cord blood, and different compartments of the placenta (Parolini et al., 2008). Since they originate from mesoderm, MSCs show *in vitro* differentiation potential into three cell lineages (adipogenic, chondrogenic, and osteogenic). These cells are highly proliferative fibroblast-like cells that display plastic adherence in culture and express specific surface markers (i.e. positive for CD105/CD90/CD73, and negative for CD34/CD45/CD11b, or CD14/CD19, or CD79 α /HLA-DR1) (Dominici et al., 2006). Importantly, MSCs are TERT-negative (Zimmerman et al., 2003). Although tumorigenicity is lower than ESC, MSCs have been shown to assist tumor growth by transformation and suppression of the antitumor immune response (Ren et al., 2009).

Similar to ESCs, MSCs display reduced immunogenicity, but MSCs also demonstrate a powerful immunomodulatory response *in vivo* (Hematti, 2008; Bifari et al., 2010). MSCs interfere with antigen-presenting cells and suppress B-cell differentiation causing inhibition of Natural Killer (NK) cells and cytotoxic T cells. They also express a broad number of anti-inflammatory factors, such as a variety of cytokines and chemokines (Banas et al.; 2008). MSCs also inhibit local and systemic proinflammatory responses through inhibition of TNF- α and interleukin (IL)-1, which functions to prevent tissue damage (Lin et al., 2011), and can nonspecifically inhibit allogeneic lymphocyte proliferation. In addition, MSCs express low levels of HLA class I antigens and lymphocyte function-associated antigen (LFA)-3, and do not express HLA class II antigens or many co-stimulatory molecules which could function to upregulate HLA class II antigens *in vivo* (Bifari et al., 2010). By far, the most exciting and potentially useful characteristics of these cells are their immunomodulatory behaviors. MSCs significantly lower the incidence of graft-versus-host disease, autoimmune diseases, and can induce tolerance upon transplantation (Le Blanc et al, 2004; 2005; 2007). Nonetheless, it should be noted that MSC therapy may also increase the vulnerability to viral infections such as herpes (Sundin et al., 2006).

3.3.1 Differentiation of MSCs and laboratory / clinical data

Similar to ESC, MSCs are able to differentiate into various cell types by stimulation with specific growth factors, and there are many published protocols for differentiating MSCs along various cell lineages. For example, a recent review describes a number of protocols for differentiating along a hepatic lineage (Puglisi et al., 2011). The ability of MSCs to differentiate into hepatocyte-like cells was first reported in 2002 (Schwartz et al., 2002). The resulting hepatocyte-like cells performed hepatic functions such as albumin production, urea synthesis, glycogen storage, and low-density lipoprotein uptake (Jiang et al., 2002).

MSCs are able to differentiate into hepatocyte-like cells by stimulation with hepatocyte growth factor (HGF), epidermal growth factor (EGF), and FGF (Lange et al., 2005). MSC-derived hepatocytes were able to engraft in the liver upon transplantation and have been shown to contribute towards improved hepatic function and regeneration (Kuo et al., 2008). Conversely, another report using bone marrow MSCs significantly increased the number of hepatic stellate cells and myofibroblasts, which could contribute to the fibrotic cascade (Russo et al., 2006). Despite the availability of many hepatic differentiation protocols, it is currently unclear whether MSCs are able to become hepatic cells through differentiation or by cell fusion, as evidenced by Sharma, et al. (2005). In addition, the homing mechanism by which intravenously injected MSCs can preferentially recruit to the injured liver is interesting (Sakaida et al., 2004), but also not well understood. MSCs preferentially target tissues undergoing remodeling; it has been suggested that inflammation might provide key regulatory factors in the targeted migration of MSCs into the diseased location (Kuo et al., 2008).

Due to their impressive immunomodulatory features, MSCs have been used to treat acute graft-versus-host disease and osteogenesis imperfecta in children (Le Blanc et al., 2005). MSCs also secrete several factors able to suppress hepatocyte apoptosis, inflammatory responses, and liver fibrosis, as well as stimulate hepatocyte proliferation and function (Lin et al., 2011; Zhou et al., 2009). Many such factors, for example HGF, can also aid in liver regeneration. Preclinical and clinical studies have suggested that MSC transplantation can moderately restore liver function and enhance survival rates in fulminant hepatic failure and end-stage liver disease (Yagi et al., 2009; Kuo et al., 2008; Banas et al., 2009), though it has been suggested that the benefits MSCs provide to damaged livers have more to do with the expression of immunomodulatory factors than engraftment and subsequent hepatic function of the transplanted cells (Banas et al., 2008). There is little evidence to date that verifies whether MSCs are able to form mature hepatocytes, either in culture or once transplanted. However, growing evidence does suggest that MSCs may improve cirrhotic liver function once infused into patients. For example, bone marrow MSCs transplantation reduced liver fibrosis, and improved liver function and survival in mice (Sakaida et al., 2004) and rats (Abdel Aziz et al., 2007). This provided rationale for the use of autologous bone marrow MSCs for cell therapy to treat cirrhosis, which spurred several clinical trials investigating cell safety and feasibility (Kharaziha et al., 2009; Mohamadnejad et al., 2007; Salama et al., 2010). At present, there are 123 ongoing clinical trials involving MSCs investigating a variety of applications including bone, cartilage, and heart repair, immune rejection and autoimmune diseases, as well as treatment for cancer, gastrointestinal, and neurodegenerative diseases (Trounson et al., 2011).

3.4 Benefits / concerns of ESCs and MSCs

Since ESCs possess the ability of unlimited self-renewal, this relieves some pressure to identify new cell sources for regenerative medicine. However, although self-renewal is generally viewed as a powerful benefit, it is also a double-edged sword. Self-renewal, genetic instability, and tumorigenicity are characteristics shared by ESCs and cancer cells. In both differentiated and undifferentiated ESCs, there is a risk of malignancy due to their associated tumorigenicity and genetic instability (Stutchfield et al., 2010). The generation of spontaneous tumors is of particular concern for clinical applications, and much of the current research is dedicated to reducing and eventually overcoming this risk. Perhaps with

more complete differentiation protocols, this will become a concern of the past. In addition to safety issues, ESCs also carry religious, political, and ethical concerns, and there is legislation restricting or banning their use in certain countries, such as the United States and United Kingdom. In contrast to ESCs, MSCs have fewer ethical concerns, as these cells are easily accessible from a variety of postnatal tissues. They exhibit a lower risk of spontaneous tumors, and their impressive ability to hide from and modulate the immune response is of considerable interest. However, MSCs have been shown to contribute to tumor growth *in vivo* and increased risk of viral infections. Therefore, high-risk patients may not be feasible candidates for MSC transplantation.

Despite many promising therapeutic reports in the literature stating stem cells are able to contribute to liver regeneration, particularly with MSCs, there are still many problems associated with their use. Even with the considerable number of differentiation protocols available to produce various cell types, differentiation methods have not been optimized. Though stem cells are abundantly proliferative, high induction rates of hepatic cells are currently not possible to provide the required number of cells for transplantation to treat disorders of the liver, an organ which contains several billion cells. Furthermore, differentiated cells display minimal hepatocyte function both *in vitro* and *in vivo*. Once transplanted, engraftment is very low with low contributions towards tissue regeneration. For these reasons, it is relatively unknown whether stem cell-derived hepatocyte-like cells will be useful to treat and correct liver disease. More research is required to determine more efficient ways to induce hepatocyte-like cells, and a standardized list of requirements should be established to verify complete differentiation. In brief, stem cell derived hepatocyte-like cells should demonstrate characteristic hepatic gene expression and function, express appropriate transport proteins and transcription factors, metabolize ammonia and bilirubin, produce albumin and/or bile acids, and no longer express genes characteristic of ESC or other cell types. Therapeutically useful hepatocyte-like cells must be safe (i.e. nontumorigenic), contribute to liver function *in vivo*, and importantly, must express hepatic genes at a level comparable to mature hepatocytes. Currently there are no definitive reports of any stem cell-derived hepatocytes with these ideal characteristics.

3.5 Human placenta as a source for stem cells

The human full term placenta is comprised of three distinct layers: amnion, chorion, and decidua (Figure 1). The amnion and chorion are fetal-derived while the decidua originates from maternal tissue. The trophoblast layer gives rise to the chorion; the amnion is derived from the pluripotent epiblast, which also gives rise to all three germ layers of the embryo. The amnion layer is established as early as day 8 following fertilization, well before gastrulation when cell fate is specified (day 15-17). The amnion is derived at a time when the epiblast remains pluripotent, and amnion epithelial (AE) cells retain some of these characteristics. AE cells are easily isolated from full term placenta following live birth, which would normally be discarded after delivery. Placenta is readily available and easily procured without invasive procedures or causing harm to either mother or baby. In 2007, there were 1.4 million cesarean births in the United States, which equates to ~32% of all U.S. births (Hamilton et al., 2009). Placentas for cell isolation are typically obtained from cesarean deliveries due to sterility concerns; however, all placentas should be considered a useful source for stem cells. Theoretically, placental stem cells could be isolated from all term births and cryopreserved in a cell bank for future use. Since it has been estimated that as little as 30

stem cell lines would be needed to match HLA haplotypes in >80% of the Japanese population (Nakatsuji et al., 2008), global banking of placental stem cells containing all HLA haplotypes worldwide may be considered a realistic and attainable goal. Therefore, placental amnion may provide a useful source of pluripotent stem cells that are plentiful and free from most ethical, religious, or political concerns.

3.5.1 Amnion epithelial (AE) cells

The amniotic layer is composed of a single-celled epithelial layer of cuboidal and columnar cells and a deeper mesodermal layer composed of an upper compact acellular layer and a lower fibroblast-containing layer (Figure 1). The epithelial layer of the amnion is in contact with the amniotic fluid, which serves to protect and cushion the fetus through gestation. The chorionic layer is comprised of a mesodermal layer and an extravillous trophoblast layer. The maternally derived decidua, which interacts with the fetal derived trophoblast, serves to support the fetus through gas, nutrition, and waste exchange, and protect the fetus from the maternal immune system.

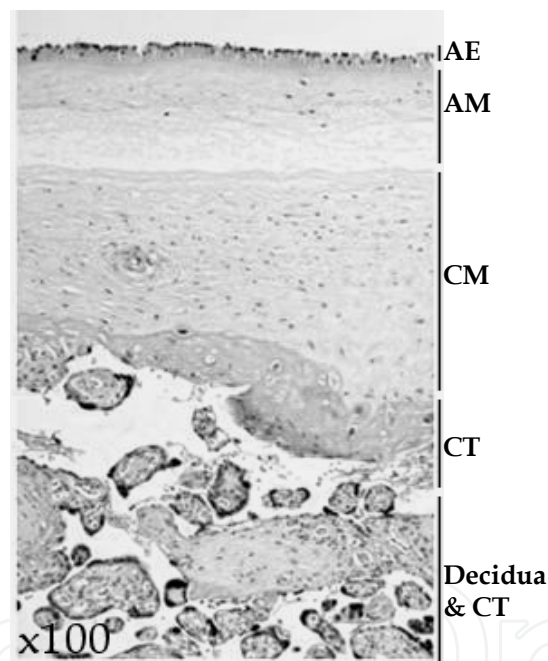


Fig. 1. Cross-sectional representation of the human placenta. The amnion and chorion are fetal-derived membranes while the decidua is maternally-derived. The amniotic layer is composed of a single-celled epithelial layer and a deeper mesodermal layer. The chorionic layer is comprised of a mesodermal layer and a trophoblast layer. The maternal decidua is intermingled with the fetal chorionic trophoblast. (AE: amniotic epithelium; AM: amniotic mesoderm; CM: chorionic mesoderm; CT: chorionic trophoblast.)

AE cells in culture express stem cell surface markers (e.g. SSEA-3 & 4, TRA 1-60 & 1-81) as well as molecular markers of stem cells (e.g. OCT-4, Nanog, SOX-2, FGF-4, and Rex-1), and unlike ESC, do not require feeder cell layers to maintain OCT-4 and Nanog expression (Miki et al., 2005; Miki & Strom, 2006). Interestingly, AE cells do not express the stem cell marker TERT (Miki et al., 2005). Telomerase activity is found in human ESC, multipotent adult

progenitor stem cells, human germ cells, and 80-90% of human tumor samples (Chen & Chen, 2011). Telomerase-positive stem cells display an unstable karyotype and can become tumorigenic, most commonly forming teratomas, when transplanted into SCID mice. Conversely, AE cells consistently display a normal karyotype and are nontumorigenic when transplanted into SCID mice (Miki & Strom, 2006; Marongiu et al., 2011). In addition, AE cells are derived from neonatal tissue and should therefore naturally possess less environmental and age-acquired DNA damage (Miki, 2011). It is commonly known in the field that amnion does not express HLA class II antigens and only expresses class I antigens at low levels, which later led to the premise that AE would be able to bypass the immune system. AE cells were also found to secrete anti-inflammatory and immunosuppressive factors, which inhibited inflammation and reduced the proliferation of T- and B-cells *in vitro* (Li et al., 2005). Volunteers transplanted with AE cells did not experience any immunological reaction, and to date no tumors have ever formed as a result (Akle et al., 1981; Yeager et al., 1985; Scaggiante et al., 1987; Sakurgawa et al., 1992). Many of these characteristics identify AE cells as similar to ESC, but not identical.

3.5.2 AE cell isolation and differentiation methods

Placental tissues are obtained with local Institutional Review Board (IRB) approval in the U.S., or under appropriate Ethical Committee approval, as well as patient approval. The amnion membrane is easily stripped from the underlying layers of the placenta by carefully peeling it away from the chorion (Figure 2). The amnion membrane contains AE cells and amniotic mesenchymal (AM) fibroblasts. AE and AM cells can be isolated from the amnion membrane following simple protocols (Miki et al., 2010; Marongiu, 2010). In brief, for collection of AE, the amnion membrane is first washed several times to remove blood contamination. The membrane is then subjected to several trypsinization steps, which releases the AE cells from the amnion mesenchymal fibroblasts and the connective tissue. The trypsin digests are then centrifuged to pellet the AE cells and resuspended in standard

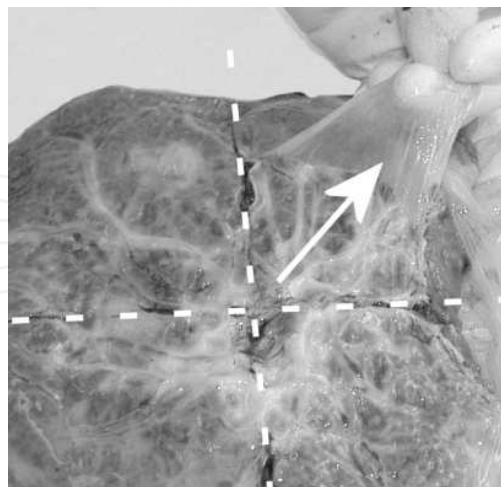


Fig. 2. Isolation of the amnion membrane from the chorion of the placenta. The maternal side of the placenta is placed face down and a shallow X-shaped incision (dashed lines) is made through the center of the placenta. The thin, nearly transparent amnion membrane is then peeled from the chorion starting at the center of the cut and progressing outward (direction of the arrow)

culture medium. A density separation step is done to enrich the population of SSEA-4 positive AE cells similar to the process used for hepatocytes. Cell viability is determined by trypan blue exclusion and counted with a hemocytometer. An estimated AE cell yield from one term placenta is 80-300 million cells (Miki et al.; 2010). When AE cells are cultured in the presence of epidermal growth factor (EGF), they readily proliferate with numerous mitotic events (Terada et al., 2000), though senescence routinely occurs after 6-10 passages (Miki & Strom, 2006).

AE cells have previously shown the potential to differentiate into all three germ layers *in vitro* (Miki et al., 2005). Similar to ESC, differentiation of AE cells to other cell types is dependent upon the culture substrate, as well as which growth factors are added and at what concentration (Parolini et al., 2007; Miki, 2011). Our group previously published efficient methods to differentiate AE cells along a hepatic lineage (Miki, et al., 2009; Marongiu, et al., 2011); after differentiation, AE cells expressed many endodermal/hepatic marker genes such as A1AT, hepatocyte nuclear factor-4 (HNF4- α), albumin, CAAT enhancer binding protein-alpha (C/EBP- α), many CYP450 genes, CK 8, 18, and 19, CYP7A1, plus several others at the level of fetal hepatocytes. Interestingly, cultured AE hepatocyte-like cells express both CYP3A7 and CYP3A4, which indicates that AE differentiates along a pathway similar to human fetal liver. Furthermore, the ratio of CYP3A4 to CYP3A7 implies that cells are progressing towards mature hepatocytes (Miki et al., 2009). Early studies demonstrated proof of principle for AE cell transplantation through the production of dopamine-expressing cells from AE, which could survive and function in a rat model of Parkinson's disease (Kakishita et al, 2000; 2003). Importantly, it was found that when undifferentiated AE cells were transplanted into the livers of immunodeficient mice, cells displaying hepatic morphology were observed that expressed mature liver genes such as transporters, cytochromes, and albumin or A1AT; circulating A1AT was also detected in transplanted mice confirming functional engraftment (Miki & Strom, 2006; Marongiu et al.; 2011).

3.5.3 AE cell transplantation

Amnion epithelial cells, due to their stem cell-like pluripotent characteristics, low immunogenicity, and anti-inflammatory properties, show exciting promise in the field of regenerative medicine. Recently, studies have shown lung protection following human AE cell transplantation in a SCID mouse model of bleomycin-induced lung injury (Moodley et al., 2010; Murphy et al., 2010). Studies have also shown the efficacy of AE cells on corneal resurfacing in horses (Plummer, 2009), rabbits (Wan et al., 2011), and humans patients (Nubile et al., 2011), in which amniotic membranes were transplanted as a graft over the injury site. These studies were done without immunosuppression and without evidence of acute rejection. Differentiated AE have also been used to treat a rat model of Parkinson's disease (Kakishita et al, 2000; 2003). Important for the treatment of liver diseases by CTx, AE cells demonstrate hepatic gene expression and functions at a level of mature hepatocytes following implantation into the livers of SCID mice, which suggest they differentiate into hepatocyte-like cells once engrafted in the liver parenchyma (Miki & Strom, 2006; Marongiu et al., 2011). Undifferentiated AE cells were able to functionally engraft into the livers of immunocompromised mouse models of liver damage resulting in a reduction of hepatic fibrosis, inflammation, and hepatocyte apoptosis (Manuelpillai et al., 2010; Marongiu et al., 2011). In addition, AE cells have also been used in clinics to correct lysosomal storage

diseases with no adverse effects (Yeager et al, 1985; Scaggiante et al, 1987; Sakurgawa et al., 1992). Finally, our group recently determined that AE cells could partially rescue a mouse model of intermediate MSUD (Skvorak et al., 2010), an inborn error of metabolism characterized by deficiency of the branched-chain keto-acid dehydrogenase (BCKDH) enzyme complex and high levels of BCAA (Homanics et al., 2006). iMSUD mice were given multiple infusions of undifferentiated AE cells, either freshly isolated or cryopreserved, directly into the liver parenchyma. AE cell transplantation partially corrected iMSUD mice similarly to the partial correction previously obtained with hepatocyte transplantation (Skvorak et al, 2009a; 2009b). While untreated iMSUD mice grew sickly and all died prior to 27 days of age, iMSUD-treated mice displayed improved BCKDH enzyme activity, reduced BCAA and other relevant metabolites in the brain and blood, their body weight mimicked that of healthy wildtype littermates, and >70% of animals survived to day of life 100 (Skvorak et al., 2010). Immunosuppression was not used and there was no evidence of rejection.

3.5.4 Benefits / concerns of AE cells

Current research suggests that stem cells isolated from discarded placenta may be an abundant, noncontroversial, and safe source of cells for regenerative medicine. There are a multitude of reports in the literature, some going as far back as 1947, which describe the successful use of AE and amniotic membranes to treat a variety of disorders both preclinically and clinically. Furthermore, isolation is relatively easy, and does not require a special laboratory set up (Miki et al., 2007). An average of 100 million cells can be isolated from a single term placenta, and AE is able to proliferate robustly in culture; Miki, et al. (2005) estimates that 100 million AE cells could be expanded to 10-60 billion cells within six passages. Unlike hepatocytes, AE cell viability and morphology are also very stable when cryopreserved long term at -80°C. Taken together, these benefits greatly encourage the establishment of a placental cell bank. Current umbilical cord blood stem cell guidelines could be used as a template to set up similar procurement and banking procedures for placental-derived stem cells (Serrano-Delgado et al., 2009).

AE cells meet many important criteria for clinically relevant cells: expression of anti-inflammatory factors, nonimmunogenic, maintains a stable karyotype, and consistently nontumorigenic *in vivo* in both SCID mice and humans. Undifferentiated AE cells are proposed to become hepatocyte-like once engrafted in the liver parenchyma and have contributed to liver function in animal models of disease. There are also many published differentiation protocols describing induction along many cell lineages, including hepatic. However, though these cells exhibit many advantages, particularly over other stem cell types, the ability to produce therapeutically useful cells to treat liver diseases has still not been developed from AE cells. Though it is unknown whether differentiation prior to transplantation will be necessary, one should assume that differentiation into the required cell type will be the most clinically efficient and effective method of treatment. Therefore, hepatocyte-like cells derived from AE should be held to the same standardized list of requirements, outlined in Section 3.4, as cells differentiated from other classifications of stem cells. As with other types of stem cells, more research is required to establish better induction of therapeutically useful cells. However, the safety of these cells has been exhaustively established and they already have a long history of clinical use. Clinical application of AE for liver and other diseases may be in our near future.

3.6 Discovery and characterization of induced pluripotent stem cells (iPSC)

Once an embryo reaches the blastocyst stage, highly specific (i.e. spatially and temporally controlled) molecular signaling events coordinate directed differentiation of cells to their appropriate cell fate. Therefore, a cell becomes specified not by changing its DNA sequence, but by controlling the expression of certain genes through specific signals. The first attempt to manipulate a cell's developmental potential was known as somatic cell nuclear transfer, which led to the birth of live lambs (Wilmut et al., 1997). "Dolly" the sheep provided evidence that differentiation of a cell towards a somatic state is not accomplished by irreversible genetic manipulation.

Almost a decade later, Yamanaka's group successfully derived pluripotent ESC-like cells from murine somatic cells through forced expression of the reprogramming factors OCT-3/4, SOX-2, c-MYC, and KLF-4 by lentiviral induction (Takahashi & Yamanaka, 2006). These "induced pluripotent stem cells" (iPSC) were similar to ESCs in morphology, growth properties, expression of ESC marker genes, display of an unstable karyotype, tumorigenicity and teratoma formation in SCID mice, and injection into a blastocyst yielded cells that contributed to mouse development. Human iPSC from adult skin fibroblasts were also generated using the same four factors as mice (Takahashi et al., 2007), and also by forced expression of a new set of four factors: OCT-4, SOX-2, Nanog, and Lin-28 (Yu et al., 2007). Pluripotent iPSC should express the stem cell markers SSEA3, SSEA-4, TRA-1-60, TRA-1-81, OCT-4, alkaline phosphatase, TERT, SOX-2 and Nanog, and form teratomas *in vivo*. It has also been reported that a specific expression profile of stem cell markers corresponds to either a completely or partially reprogrammed cell (Chan et al., 2009). Recently, iPSCs have been derived from a variety of human tissues, such as umbilical cord matrix (Cai et al., 2010), fetal and juvenile tissues (Park et al., 2008a; Li et al., 2010; Aasen et al., 2008), placental tissue (Nagata et al., 2009; Cai et al., 2010; Zhao et al., 2010), and primary human hepatocytes. However, evidence that iPSC retain epigenetic memory of their cells of origin exist for both mouse (Kim et al., 2010; Polo et al., 2010) and human (Hu et al., 2010), which can affect their differentiation potential. This is a concern, but it also suggests that the best cell source to generate iPSC for the treatment of liver disease would be hepatocytes; hepatic-like cells differentiated from hepatocyte-derived iPSC would most closely resemble their primary cell counterparts.

Currently, iPSC research is largely dedicated to reducing the tumorigenicity of the cells. The use of retroviruses and lentiviruses is a concern, which integrates into the target cell genome in a random fashion potentially causing cancer. The generation of mouse iPSC through the use of non-integrating adenoviruses (Stadtfield et al., 2008) determined reprogramming could be achieved through transient expression. Vector integration-free human iPSCs have since been derived using Epstein-Barr virus-derived episomes (Yu et al., 2009), mRNA transfection (Yakubov et al., 2010), bacterial DNA-free episomal vectors (Jia et al., 2010), and proteins (Kim et al., 2009). However, viral integration is only one problem contributing to the tumorigenicity of iPSC. c-MYC is a well established oncogene, and the remaining factors (OCT-4, SOX-2, KLF-4) are also known to be highly expressed in cancers (Schoenhals et al., 2009). Recent studies have shown that reprogramming could be successful without using c-MYC and KLF-4 (Li et al., 2010; Huangfu et al., 2008). However, the use of viral-free vectors or the omission of c-MYC and KLF-4 drastically reduces reprogramming efficiency, and one study found no difference in tumorigenicity between viral and viral-free methods

(Moriguchi et al., 2010). More recently described epigenetic factors such as cell memory and genetic imprinting may also contribute to the tumorigenicity of iPSC, which is not yet understood (Ben-David & Benvenisty, 2011).

3.6.1 iPSC for disease modeling, hepatic differentiation, and transplantation

Now that iPSC technology has been well established, disease-specific iPSC to model diseases *in vitro* and *in vivo* may help researchers better understand diseases in order to develop new treatments. Park et al. (2008b) described the generation of iPSC from a variety of inherited diseases, such as Huntington's, Duchene's and Beckers's muscular dystrophy, diabetes mellitus type 1, Down's syndrome, and Parkinson's. iPSC have also been generated from inherited metabolic disease patients with A1AT, Crigler-Najjar, tyrosinemia type 1, familial hypercholesterolemia, and glycogen storage disease type 1a (Rashid et al., 2010), which were then differentiated into hepatocytes to more accurately model the disease *in vitro*. More recently, iPSC-derived hepatocyte-like cells generated from the dermal fibroblasts of a Wilson's disease patient was shown to mimic the disease phenotype *in vitro* (Zhang et al., 2011). Importantly, iPSCs of metabolic disease could be genetically corrected in culture, differentiated into hepatocytes possessing the ability to make normal protein, and potentially infused back into patients to cure their disease. The use of autologous cells would also reduce the risk of immune issues and rejection theoretically avoiding the need for immunosuppression. Human artificial chromosome technology was used to deliver the entire dystrophin gene to genetically correct patient fibroblasts with Duchenne muscular dystrophy *in vitro*, and iPSC were generated from the corrected cells (Kazuki et al., 2010). In addition, iPSC-derived hepatocyte-like cells modeling Wilson's disease were corrected *in vitro* using either lentiviral gene therapy or treatment of the drug curcumin (Zhang et al., 2011).

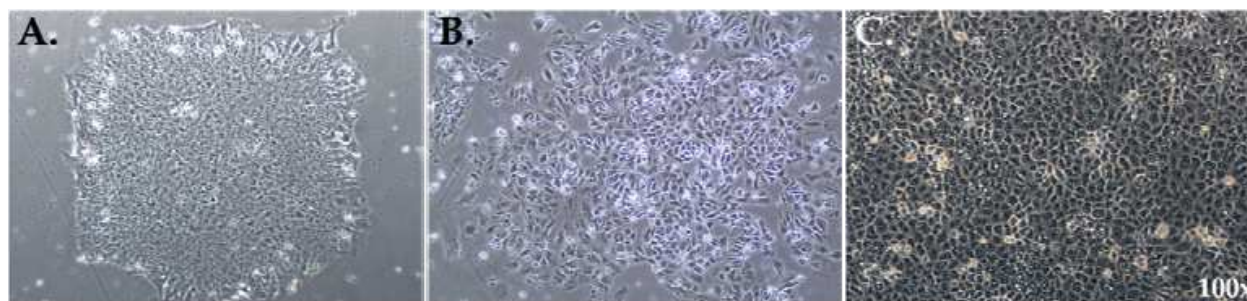


Fig. 3. Examples of iPSC *in vitro*. A. undifferentiated iPSC cell colony; B. a spontaneously differentiating iPSC cell colony; C. iPSC cell-derived hepatocytes formed after following an *in vitro* protocol adapted from Si-Tayeb et al. 2010. All cells were cultured on matrigel in mTeSR1 media

Using established differentiation protocols for ESCs, researchers have been able to differentiate iPSCs into all three germ layers in culture, further proving these cells are truly pluripotent. Figure 3 shows examples of cultured iPSC in either an undifferentiated state (Figure 3A) or spontaneous differentiation (Figure 3B). Furthermore, there are several reports of differentiation of iPSC along a hepatic lineage (Figure 3C) using ESC protocols (Si-Tayeb et al., 2010; Song et al., 2009; Liu et al., 2011). However, in most reported cases iPSC-derived hepatocytes displayed very low hepatic function and gene expression *in vitro* when compared to primary hepatocytes. However, blastocysts from tyrosinemia type 1

mouse models deficient in the FAH gene were injected with MEF-derived iPSC, and resulting pups with high chimerism could survive without NTBC drug (Espejel et al., 2010). FAH-negative pups with low levels of chimerism were dependent on NTBC to survive. These data demonstrate the potential for iPSC to form “mature” hepatocytes which can express sufficient levels of protein to correct a metabolic defect. However, Espejel et al. differentiated iPSC through injection into a blastocyst. As with other stem cells, improved *in vitro* differentiation protocols are needed to yield high amounts of therapeutically useful cells.

3.6.2 Benefits / concerns of iPS cells

Similar to ESC, the major benefits of iPSC are their self-renewal abilities and differentiation potential. iPSC could theoretically provide an unlimited pluripotent source of cells that could be banked and differentiated into hepatocytes for transplant when needed. Nakatsuji et al. (2008) estimated that an iPSC bank with only 30 stem cell lines could match the HLA haplotypes in >80% of the Japanese population. Unlike ESC, patient specific iPSC could be generated, corrected, and infused back into a patient avoiding immune problems and the need for immunosuppression. Importantly, there are no religious, ethical, or political controversies associated with the use of iPSCs. However, the field of iPSC research is very new and there are still a lot of unknowns. The major concern with iPSC use is a question of safety; iPSC have a high risk of tumorigenicity. Just like ESC, iPSC exhibit genetic instability, express TERT, and can produce teratomas *in vivo*. Furthermore, rapidly accumulating evidence suggests these two cell types have important genetic and epigenetic differences that influence their tumorigenicity, and that iPSC are likely more tumorigenic than ESC (reviewed by Ben-David & Benvenisty, 2011). Furthermore, the most reliable, reproducible, and efficient method to currently generate iPSC is through an integrating viral vector process that could induce cancers, and both integrating and non-integrating methods use reprogramming factors that are highly expressed in many tumor samples. Aside from the issue of safety, cell differentiation protocols and methods to enhance engraftment have not yet been optimized, and it is currently unknown whether long term survival of iPSCs *in vivo* is possible. Further research is needed to generate iPSC that are safe, effective, and therapeutically useful before these cells can be used for clinical cell therapies.

4. Conclusions

Alternatives to OLT must be found in order to circumvent the increasing amount of patient deaths due to long organ wait times and insufficient numbers of available livers. CTx has shown a great deal of promise, and the progress made over the past several decades of preclinical and clinical studies provides a growing amount of rationale for its use to treat a variety of liver disorders. Cells isolated from donor livers have been proven to provide safe and effective liver support for both short- and long-term function. There have been several reported clinical cases of disease reversal in acute liver failure, and HTx has provided partial correction for a variety of inherited metabolic diseases (Table 1). *In vitro* gene modification to correct allogenic hepatocytes is also possible to avoid immunogenicity of transplanted cells and a lifelong immunosuppression regimen. However, complete correction of a metabolic disorder by HTx has not yet been achieved, and more than one treatment would likely be required to sustain a patient through his/her lifetime. There are still several

challenges to overcome. For HTx, the first major challenge is the availability of donor livers for the isolation of hepatocytes. There are not enough donor livers rejected for OLT, thus making them available for HTx, to provide for everyone requiring treatment. The second is regarding reliable storage of isolated hepatocytes. Current cryopreservation and thawing protocols result in massive cell loss and decreased viability; this reduces the number of cells available for transplant, and cell quality influences cell engraftment, cell function, and thus patient outcome. These drawbacks emphasize our need for alternative cell sources. Research to use stem cells and stem-like cells (e.g. ESC, MSC, AE, iPSC) for CTx are currently in preclinical and, for some, early clinical stages. Though there have been some advances made in animal models, the safety and efficacy of these cells must be unequivocally determined. All the aforementioned alternative cell types display varying levels of immunomodulatory properties making them potentially less immunogenic than hepatocytes. However, ESC, MSC, and iPSC all have tumorigenicity risks associated with their use *in vivo*. It has been suggested that more complete differentiation of cells into hepatocyte-like cells may reduce tumor formation, though it is not known whether this strategy will completely ablate the risk. In addition, the recently discovered epigenetic factors in iPSC must be more thoroughly investigated. These epigenetic differences may influence differentiation efficiencies, and it has been suggested that they may make iPSC more tumorigenic than ESC (Ben-David & Benvenisty, 2011). AE cells are nontumorigenic in both an undifferentiated and differentiated state, are nonimmunogenic, and have been used in clinical studies for more than sixty years without immunosuppression and without evidence of acute rejection. AE cells are clearly the safest alternative to hepatocytes from the stem cells discussed in this chapter, but their effectiveness to correct liver disease is currently unknown.

In summary, considerable progress has been gained in cell transplantation thus far, though future work is required to enhance utility of this novel branch of regenerative medicine. Improvement of cell engraftment remains the single biggest challenge to overcome. New methods to modulate the immune reaction and relieve changes in vascular pressures after cell transplant are currently being investigated to enhance engraftment and improve patient outcome. Preconditioning protocols of the recipient liver, such as hepatic irradiation, portal vein embolization, and surgical resection, may also help to improve engraftment by giving donor cells selected growth advantage (Soltys et al., 2010; Puppi et al., 2011), which will be of particular importance in patients of metabolic disease. Though hepatocytes remain the most preferred cell for cell transplantation, stem cells may provide a useful cell alternative to hepatocytes once the question of safety has been resolved and the ability to provide therapeutically useful cells at a scale suitable for transplantation is achieved.

5. Acknowledgements

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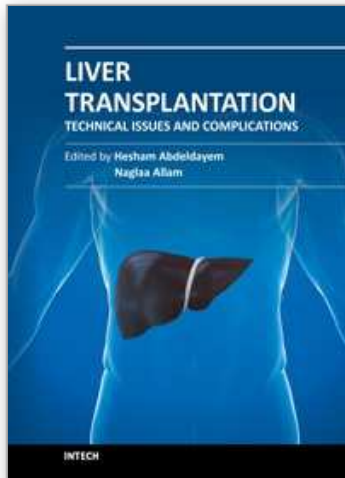
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This book covers a wide spectrum of topics including, but not limited to, the technical issues in living and deceased donor liver transplant procedures, cell and experimental liver transplantation, and the complications of liver transplantation. Some of the very important topics, such as the arterial reconstruction in living donor liver transplantation, biliary complications, and the post-transplant-lymphoproliferative disorders (PTLD), have been covered in more than one chapter.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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