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Stem cell approaches to supply hepatocytes for regenerative medicine and in vitro pharmaceutical testing systems (literature review)

‘This thesis is the result of the author’s original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.’

Signed: Date: 13th of August 2012

Abstract

The liver is one of the most complicated and essential organs in the human body. Its complexity has made it impossible to replicate its functions artificially. Therefore, when the liver fails to function correctly a liver transplant is the only current solution. There would be more transplants but for the lack of donors. The combination of liver diseases with the lack of liver donors results in thousands of deaths in the UK every year. To help bridge the gap to transplantation or heal the damaged liver, fully functional hepatocytes are required. When trying to replace liver function the lack of functional human hepatocytes currently available is the biggest problem facing cellular therapies and bioartificial liver devices. Human hepatocytes quickly lose their metabolic functions after their host dies and are difficult to cryopreserve whilst animal hepatocytes are usually avoided due to fear of spreading viral diseases. This literature review investigated the use of stem cells as a suitable cell source to overcome the lack of functional hepatocytes currently available. Human embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and mesenchymal stem cells have the potential to produce an unlimited supply of hepatocytes. An unlimited supply of fully functioning hepatocytes would be invaluable for cellular therapies, liver tissue engineering, bioartificial liver devices, modelling inherited and chronic liver diseases as well as drug development and toxicity testing. Differentiating stem cells (taken from anywhere other than the liver) into mature, fully functional hepatocytes would get around the lack of liver donations. ESCs, iPSCs and MSCs were examined in terms of differentiation procedures, resulting cells, advantages, disadvantages and any related concerns.

Contents

1. Introduction

The liver carries out over five hundred functions and is one of the most complicated and essential organs in the human body. The liver's complexity has made it impossible to replicate its functions artificially unlike the major function of the kidneys which can be duplicated by a dialysis machine. Therefore, when the liver fails to function correctly a liver transplant is the only current solution. There are approximately 600 to 700 liver transplants (6,599 between 2000 and 2010) carried out annually in the UK [1]. There would be more transplants but for the lack of donors. In the last twenty years the number of liver donations has remained fairly constant but the amount of people who would benefit from a transplant has increased by an estimated 90% [2]. The amount of people requiring a liver transplant therefore far exceeds the number of liver donations available [2]. As a result of this discrepancy, liver disease is responsible for thousands of deaths in the UK every year (16,087 in 2008) [2]. When trying to replace liver function the lack of functional human hepatocytes currently available is the biggest problem facing regenerative medicine and bioartificial liver devices. The metabolic function of hepatocytes quickly degenerates after a person's death as soon as blood circulation to the organ ceases. As stated above there is already a severe lack of liver donations so those available are currently used in conventional transplants. Human hepatocytes are difficult to cryopreserve and animal hepatocytes are usually avoided due to fear of spreading viral diseases. It is not just cellular therapies and bioartificial liver devices that require fully functioning hepatocytes; they would also be a great asset to the pharmaceutical industry. A major restraint of human in vitro studies is the shortage of primary human hepatocytes [3] [4]. The hepatocytes usually used in testing drugs tend to come from small sections of liver removed for medical reasons [3]. Drug induced liver injury is one of the main reasons why new drugs are rejected instead of approved and why approved drugs are withdrawn from the market [4]. A renewable source of hepatocytes would allow new drugs to be safely tested [3]. The liver is crucial to the metabolic function of most drugs [3]. Some of the developed in vitro human liver models include supersomes, perfused lines, microsomes, S9 fraction, cytosol, cell lines and liver slices [3]. These models have been proven somewhat useful but suffer functional drawbacks [3]. Animal models are also used for testing new drugs. Liver failure is induced in dogs using paracetamol and in rabbits using galactosamine. Surgically induced liver failure is also used on dogs, rabbits and pigs. All of these animal models have downsides including the ethical implications associated with killing animals. In clinical trials more than

half of all the drugs that are hepatoxic to humans do not cause liver injury in animals [4]. One of the best ways of predicting the effectiveness and toxicity of new drugs would be to have a consistent in vitro test model [3]. This literature review will investigate the use of stem cells as a suitable cell source to overcome the lack of functional hepatocytes currently available. It will cover three types of stem cell: embryonic, induced pluripotent and mesenchymal.

2. Liver Failure and Liver Transplants

2.1 Liver Damage and Liver Diseases

Liver damage can be caused by alcohol, infection or illness [2]. The liver is an extremely tolerant organ and if left disease free, can function properly into old age [5]. Often liver disease shows unclear symptoms or more often, no symptoms at all [5]. The three main causes of liver failure are chronic damage (inflicted over several months or years), acute damage (inflicted over hours or days) and liver cancer (cancer that originates in or spreads to the liver) [6].

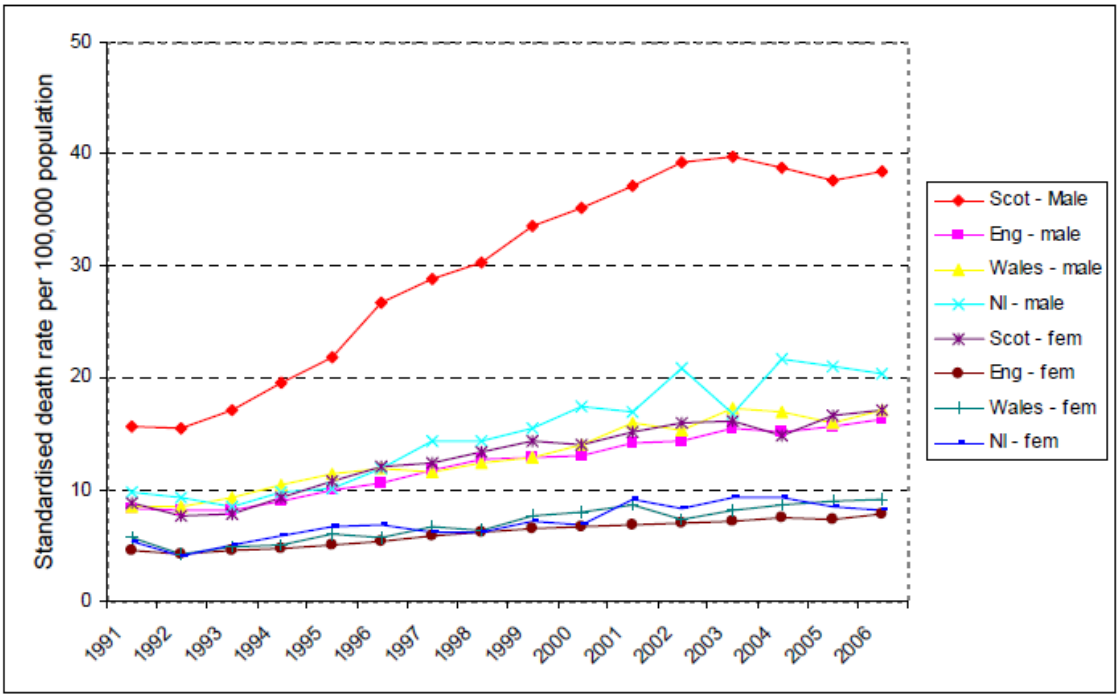
2.2 Chronic Liver Damage

2.21 Alcoholic Liver Disease

Alcohol is toxic and each time someone drinks alcohol, their liver has to filter out the poisonous alcohol from the blood [6]. Every time the liver is exposed to alcohol, some of its cells die. However the liver is capable of regenerating replacement cells [6]. Consequences only arise when a person drinks too much for too long (several years) because then the liver loses its ability to regenerate functional cells and instead scarring is formed [6]. This scarring is called cirrhosis [6]. When the liver has too much cirrhosis and not enough healthy cells, it stops functioning properly [6]. This results in liver failure [6]. Another complication associated with drinking heavily over an extended period of time is called "fatty liver" where the liver cells are swollen with surplus fat [5]. The factors contributing to Alcoholic Liver Disease are not as simple as some may think; not everyone who indulges in too much alcohol develops liver damage [5]. No one knows for sure why only certain people develop Alcoholic Liver Disease from drinking too much alcohol but some believe it is due to an amalgamation of factors including genetics [5]. Alcoholic Liver Disease is usually symptomless but when the disease develops it causes an acute illness [7]. This illness immediately kills between a quarter and a half of the people infected [7]. The lack of symptoms is very unfortunate because many people are oblivious to the damage they are doing to themselves [6]. The misuse of alcohol is estimated to cost the NHS £2.7 billion every year [7]. In 2007, Alcoholic Liver Disease killed 4,580 people in England and Wales

[7]. The period between 1999 and 2005 saw a 41% increase in Alcoholic Liver Disease deaths [7]. There was a 450% increase in the number of deaths from Alcoholic Liver Disease between 1978 and 2008 [7]. In Scotland during the 2006/07 financial year 1,094 children (under 18 years old) were admitted to hospital with diagnoses related to alcohol whilst during 2007/08 there were 6,817 patients discharged from hospital with Alcoholic Liver Disease [7]. This was a 400% increase on the 1996 statistics [7]. Treating alcohol related problems costs Scotland over £1 million every day [7]. The alcohol related mortality in the UK between 1991 and 2006 is shown in Figure 1 [8]. The increase in alcohol mortalities is particularly worrying for Scottish males. The high number of alcohol related deaths is not only a British problem, roughly 79,000 deaths occur every year due to excessive alcohol in the United States [9]. 15,183 are alcoholic liver disease deaths [10].

Figure 1. Alcohol related mortality in the UK between 1991 and 2006 [6]



2.22 Primary Biliary Cirrhosis (PBC)

Primary Biliary Cirrhosis (PBC) is another type of liver disease that causes chronic liver damage [6]. It is believed that this is caused by a build up of bile within the liver. The bile builds up because the sufferer's own immune system damages the bile ducts that usually secrete the bile out of the liver [5] [6] [11] [12]. This can eventually lead to cirrhosis [5]

[12]. PBC is quite rare and affects approximately 1 in every 8,300 people in the UK [6]. However it is responsible for the need for a large number of transplants [6]. It was reported in the Birmingham transplant unit that a quarter of those undergoing a liver transplant suffered from PBC [6].

2.23 Hepatitis C

Hepatitis C (HCV) can be caught by coming into contact with a person already infected with the disease [5] [6]. For this reason Hepatitis C is known as a blood borne virus [5] [6]. It can also be spread during contact with other body fluids although this is less common [6]. Before blood donations started being screened for Hepatitis C and Hepatitis B in September 1991 it was possible to catch Hepatitis C from blood transfusions and other blood products [5]. Nowadays the people who are at most risk are drug users who share needles [5] [6]. Just under 40% of intravenous drug users have HCV and about 35% of people infected with HCV have contracted it through sharing needles [11]. It can also be passed on by unsterilized tattoo or piercing equipment, sharing a toothbrush or razor blade and through sex although the latter is rare [11]. It is estimated that more than 250,000 people are infected with Hepatitis C in the UK [11]. Some estimate that as many as 466,000 people have HCV in the UK [7]. Only a very small amount of these infections have been confirmed by laboratories (7,540 diagnoses in 2007) [7]. 80% of people with Hepatitis C are unaware that they are infected because they have no symptoms [11]. About 20% of people with HCV will naturally get rid of the virus [7]. Approximately 75% of the people with HCV will develop chronic Hepatitis C [11]. HCV can cause scarring and swelling of the tissue that makes up the liver [6]. This can lead to liver damage [6]. Around 15% of the people who contract Hepatitis C will have liver failure [6]. This often happens decades after the initial HCV infection [6]. In the UK, the number of people with end stage liver disease related to Hepatitis C continues to increase [7]. The number of new cases doubled between 1996 and 2005 [7]. By 2015 the number of new cases per year is predicted to increase to 2,670 and the number of people with Hepatitis C related cirrhosis is expected to more than double to 8,280 [7]. There is no vaccine for Hepatitis C but there is an effective treatment for it [11]. Under 30% of diagnosed patients were treated with NICE approved antiviral therapy in 2007 [7]. In the next 30 years it is estimated that HCV will cost the NHS up to £8 billion [7].

It is estimated that 3% of the world's population (around 170 million people) has chronic Hepatitis C with up to 4 million new cases every year [7] [11].

2.24 Hepatitis B

Hepatitis B (HBV) is another blood borne virus spread by sharing needles and during sex. Hepatitis B is not very common in the UK. Approximately 1 in 1,000 people have HBV with between 2 % and 10% of them developing chronic infection [6]. If this long term infection is left untreated roughly 15% to 25% of these people will develop liver failure [6]. There is a vaccine that can protect against HBV [6]. However not everyone has access to this vaccine and around 2 billion people have been exposed to Hepatitis B worldwide [5] [7]. Worldwide over 350 million people have chronic or lifelong Hepatitis B infections [7]. Every year Hepatitis B takes the lives of between 500,000 and 700,000 people through liver cancer or liver cirrhosis [7]. 75% of the world's population live in regions of high infection [5]. It is very common in Africa, South East Asia, the Middle and Far East as well as in Southern Europe [13].

2.25 Hepatitis A

Usually sufferers of Hepatitis A recover with no long term damage to the liver [5]. It is only Hepatitis B and C that cause long lasting liver disease which can lead to cirrhosis and possibly cancer [5].

2.26 Primary Sclerosing Cholangitis

Primary Sclerosing Cholangitis (PSC) affects the bile ducts by making them narrower due to scarring and inflammation [5]. This condition results in a bile build up which causes long term inflammation of the liver [6]. After many years this can result in liver failure [6]. PSC is rare in the UK and affects around 1 in 16,000 people [6].

2.27 Autoimmune Hepatitis

Autoimmune Hepatitis is very rare and affects mostly women [5] [6]. It usually occurs just after the start of puberty or menopause [5]. For some unknown reason the sufferer's own white blood cells start attacking the liver which results in damage and inflammation [6]. This can lead to liver failure [6]. The condition can be suppressed with drugs and affects about 1 in 100,000 people in the UK per year [5] [6].

2.28 Biliary Atresia

Biliary Atresia is a rare condition that affects babies [6]. The babies are born with blocked bile ducts which leads to a bile build up (similar to PBC) that can eventually cause liver failure [6]. In the UK it affects 1 in 18,000 births and is the leading reason for childhood liver transplants [6].

2.3 Acute Liver Damage

Acute liver failure is much less common than chronic liver failure [6]. Around 1 out of 300,000 people is affected by acute liver failure each year in the UK [6]. In the developed world, acute liver failure only affects between 1 and 6 people out of a million every year [14]. Unlike chronic liver failure, most cases of acute liver failure occur in younger adults [6]. Virally induced acute liver failure has greatly decreased in the USA and Western Europe in recent years [14]. Drug induced liver failure is the most common cause in the USA and Western Europe [14]. However in the developing world viral infections such as Hepatitis E are the most common cause of acute liver failure [14]. Figure 2 shows a breakdown of selected countries and what causes acute liver failure in that country [14]. The most common cause of acute liver failure in the UK is accidently or purposively overdosing on paracetamol [6]. High levels of paracetamol can have an extremely toxic effect on the liver [6]. Paracetamol causes liver damage because it is converted by hepatic cytochrome P450 enzymes into a toxic metabolite (N-acetyl-p-benzoquinoneimine) [15]. In vivo studies have proven that patients are more susceptible to paracetamol induced liver damage when these enzymes are stimulated before administering the inducing agents [15]. Some other,

rarer causes of acute liver failure include hyperthermic injury caused by heat shock or protracted seizures, Amanita species poisoning (mushroom poisoning), Wilson's disease (genetic disorder that results in copper building up in the liver) and ischemic injury due to systemic hypotension [14].

Figure 2. Selected countries and their causes of acute liver failure [14]

	Drug		Viral			Unknown	Other
	Paracetamol	Non-paracetamol	HAV	HBV	HEV		
Spain 1992–2000[18]	2%	17%	2%	32%	..	35%	12%
Sweden 1994–2003[19]	42%	15%	3%	4%	..	11%	25%
UK 1999–2008[20]	57%	11%	2%	5%	1%	17%	7%
Germany 1996–2005[21]	15%	14%	4%	18%	..	21%	28%
USA 1998–2001[22]	39%	13%	4%	7%	..	18%	19%
Australia 1988–2001[23]	36%	6%	4%	10%	..	34%	10%
Pakistan 2003–05[24]	0%	2%	7%	20%	60%	7%	4%
India 1989–96[25]	0%	1%	2%	15%	44%	31%	7%
Sudan 2003–04[26]	0%	8%	0%	22%	5%	38%	27%

..=not reported. HAV=hepatitis A virus. HBV=hepatitis B virus. HEV=hepatitis E virus.

2.4 Liver cancer

There are two main categories of liver cancer: secondary and primary. Secondary liver cancer (sometimes called metastatic cancer) develops elsewhere in the body and then spreads to the liver [16]. Primary liver cancer starts in the liver [6] [16]. The two main types of primary liver cancer are: Hepatoma which is also known as Hepatocellular Carcinoma (HCC) and Biliary Tree Cancer which includes bile duct cancer and gallbladder cancer [16]. In the UK there are an estimated 3,000 new cases of primary liver cancer each year making up around 1% of all UK cancers [5] [16]. Most of these people have cirrhosis which may have developed from a selection of causes [5]. Secondary liver cancer is much more common than primary liver cancer [16]. The risk factors associated with liver cancer include: alcohol misuse, HBV or HCV and obesity (when a person's BMI is 30 or more) [17]. The rates of liver cancer are increasing in the UK due to an increase in alcohol misuse, obesity and type 2 diabetes [17]. In a lot of cases, liver cancer does not cause any noticeable symptoms until it has reached an advanced stage [17]. This results in only 1 in 10 people being diagnosed at an early stage [17]. The majority of people who are diagnosed with liver cancer cannot be cured because the disease is so advanced [17]. After

diagnosis just 1 in 5 people live for at least a year and only 1 in 20 people live for at least five years [17]. Liver cancer is the 3rd most prolific killer of all the cancers worldwide [17]. 80% of liver cancer cases occur in the developing world [17]. Primary liver cancer is pretty rare in the UK; it is much more common elsewhere [16]. Hepatocellular Carcinoma is the 6th most common cancer in the world [16]. It is particularly common in parts of Asia like Mongolia and in sub-saharan Africa [16] [17]. More than half of all cases of liver cancer occur in China [17]. The regional concentration of liver cancer cases is due to the lethal combination of HBV and aflatoxins [17]. As stated earlier, HBV is very common in parts of Asia and Africa [17]. Aflatoxins are toxic metabolites that contaminate food and are also found in East Asia and Africa [17] [18]. Aflatoxins occur in raw agricultural products including crops prior to harvest and after harvest (if crop drying is postponed, the excess water promotes mould growth) [18]. They occur in peanuts, cheese, corn, milk, almonds, spices and figs [18]. If animals are fed with food contaminated with aflatoxins, this can lead to them producing contaminated milk, eggs, and meat [18]. Corn and peanuts have the highest risk of aflatoxin contamination [18].

2.5 Liver Transplants

When cirrhosis of the liver passes a certain point the liver steadily loses its ability to function correctly [5]. Liver failure is when the liver can no longer complete its functions [2]. The liver's complexity has made it impossible to replicate its functions artificially. As a consequence when the liver fails, a liver transplant is the only current solution. During a liver transplant the diseased or damaged liver is removed and a healthy, fully functional one is implanted. Liver transplants are still regarded as a major operation with one of the highest risks being that the body will reject the healthy liver [2]. To avoid this rejection anyone who undergoes a liver transplant has to take immunosuppressants for the rest of their lives [2]. There are currently three forms of liver transplant: deceased organ donation, living donor organ transplant and a split donation [2]. A deceased organ donation is the most common and involves implanting a liver taken from a recently deceased person [1]. A living donor organ transplant takes a section of liver from a living donor (usually a blood relative) and uses it to replace the unhealthy liver [19]. The liver's amazing ability to regenerate results in both the sections of the donor's liver growing to normal size [19].

Initially this seemed like a breakthrough idea capable of preventing deaths from liver failure due to the lack of deceased organ donations however there were too many complications with donors [19]. An average of 16% of living donors experienced problems affecting the liver, small bowel, lungs or bleeding [19]. This has resulted in expert advisers expressing concerns about the risks facing donors [19]. They explained that some of the living donors may end up needing a liver transplant of their own after a while [19]. It was also recorded that the risk of death increased with the size of the donated liver lobe [19]. A split donation takes a deceased organ donation and splits it into the large right lobe and the smaller left lobe [2]. The right lobe is inserted into a fully grown adult whilst the left lobe is implanted into a child or very small adult [2]. Just like with a living donor organ transplant, both sections of liver should regenerate into full sized livers [2]. Figure 3 shows how many liver transplants have been carried out in Europe between May 1968 and December 2010 [20]. Figure 4 shows how the number of liver transplants carried out annually in Europe increased between 1968 and 2008 [20].

Figure 3. The breakdown of European liver transplants between May 1968 and December 2010 [20]

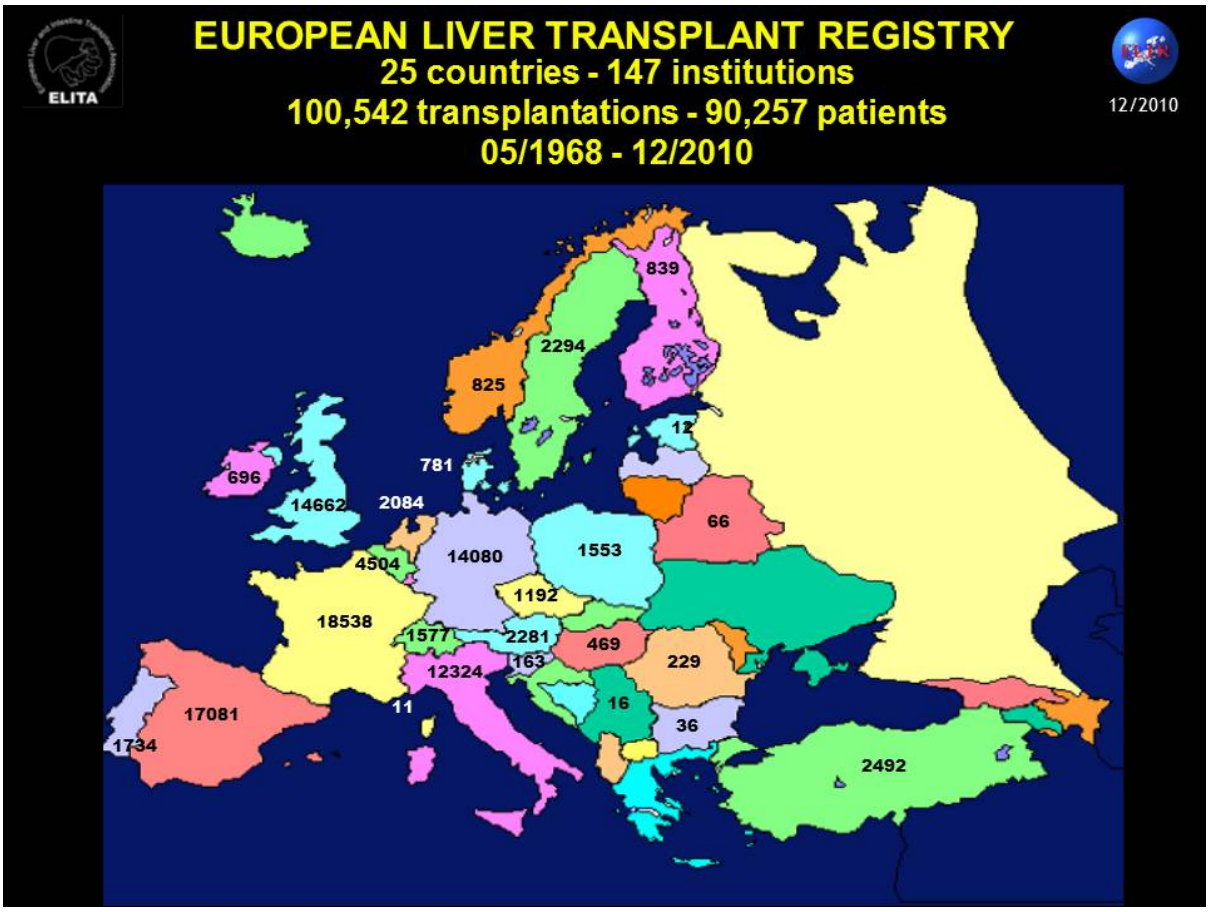
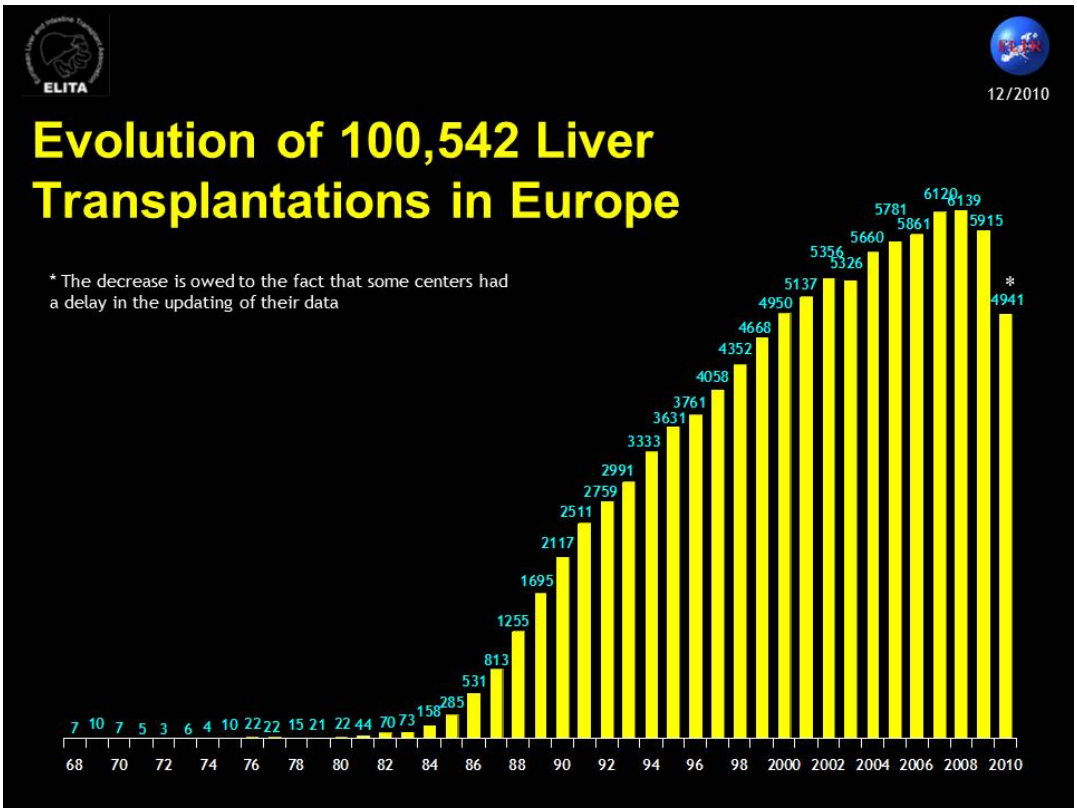


Figure 4. The number of European liver transplants carried out annually between 1968 and 2009 [20]



2.51 UK Liver Transplant Statistics

There are approximately 600 to 700 liver transplants (6,599 between 2000 and 2010) carried out annually in the UK [1]. Figure 5 displays the number of deceased donors, transplants and patients still on the active waiting list each year between 1st April 2000 and 31st March 2010 in the UK [21]. For example, in the 2007/08 financial year there were 1,121 patients on the liver transplant waiting list [7]. As can be seen from Figure 5, there were 633 transplants and 268 people still waiting for a transplant on the 31st of March 2008 meaning that a staggering 220 were removed from the waiting list in that year due to death or variations in health [7]. In the 5 year period between 2002 and 2007 there were 3333 liver transplants [7]. In this period the top three reasons for a liver transplant were: cirrhosis due to Alcoholic Liver Disease with 527 patients, 412 patients with cirrhosis related to Hepatitis C and Primary Biliary Cirrhosis with 281 patients [7]. In the 2008/09 financial year there were 667 deceased donor liver transplants with 537 whole liver transplants and 107 split transplants [1]. In this year alcohol was the most prolific cause for

a liver transplant with 112 transplants [1]. Next was Hepatitis C with 107 transplants and the third most common was Primary Biliary Cirrhosis with 58 transplants [1]. Around 88% of people are still alive a year after their liver transplant [1]. Today's survival rate is far superior compared to those who initially underwent a liver transplant in the 1960s [1]. Better immunosuppressants have played a crucial role in this improvement [1]. However they do have a downside; possibly causing side effects and making people increasingly susceptible to disease and infection [1]. The post registration outcome of 569 new adult elective liver only (not combined with kidney, bowel or pancreas transplantation) registrations made in the year between 1st April 2007 and 31st March 2008 in the UK is shown in Figure 6 [21]. There are a total of 7 liver transplant units in the UK: 6 in England and 1 in Scotland [2]. Obviously there would be more transplants but for the lack of donors [2].

Figure 5. Number of deceased donors, transplants and patients still on active transplant list in the UK between 1st April 2000 and 31st March 2010 [21]

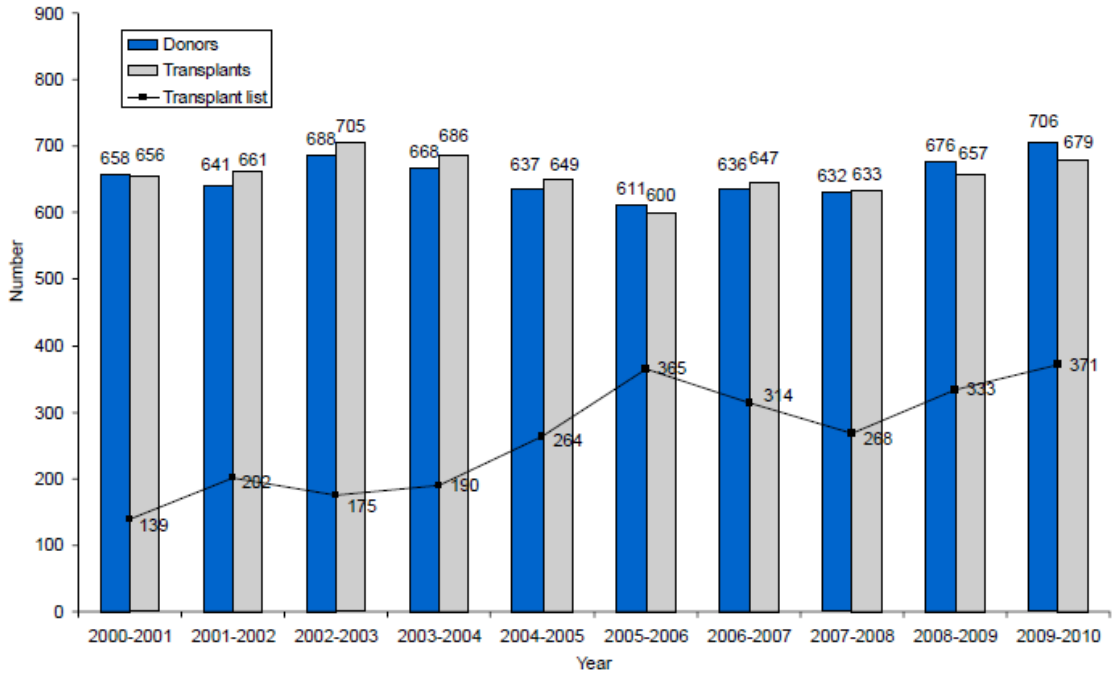
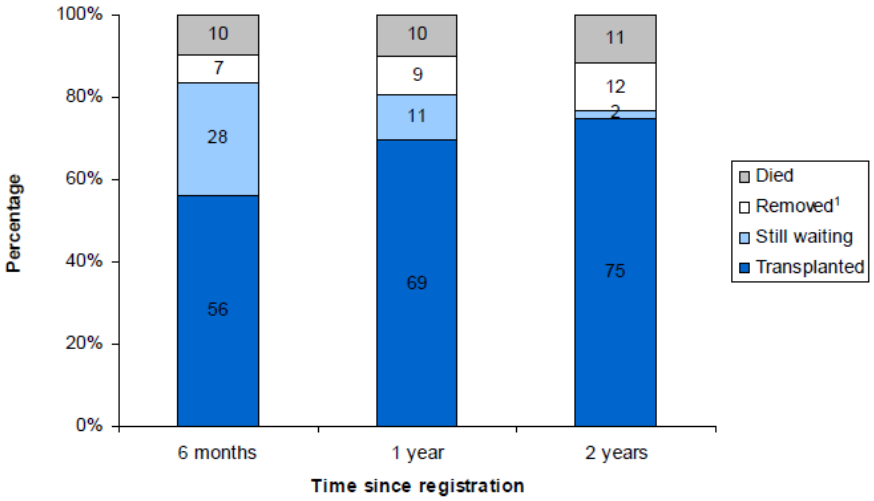


Figure 6. Post registration outcome of 569 new adult elective liver only registrations made in the UK between 1st April 2007 and 31st March 2008 [21]



[1] Includes removals due to condition deteriorated

2.52 Rest of the World Liver Transplant Statistics

The shortage of liver donations is not just a problem in the UK; it is also a big problem in the USA. Figure 7 shows the number of people still waiting for a liver transplant in the USA at the end of the calendar year between 2002 and 2008 [22]. Figure 8 shows the number of patients alive on the waiting list at any time during the year as well as the annual drop out (removal due to death or becoming too sick) rates from liver waiting list per 1000 patient-years [22]. In 2010, there were 6,600 liver transplants carried out in the EU however the ratio of operations compared to people on the waiting list was 2:3 [23]. Around 11,000 liver transplants are carried out annually all over the world and most centres are reporting an increase in their liver transplant waiting list mortalities with a worldwide waiting list mortality rate of between 15 and 30% [24]. Spain has roughly twice as many organ donors (32 per million inhabitants) compared to the UK (16.4 per million inhabitants) and the EU average (16.5 per million inhabitants); the risk of mortality on the liver transplant waiting list in Spain is between 6 and 10% [23] [25].

Figure 7. Number of candidates on the liver waiting list at the end of the calendar year in the USA [22]

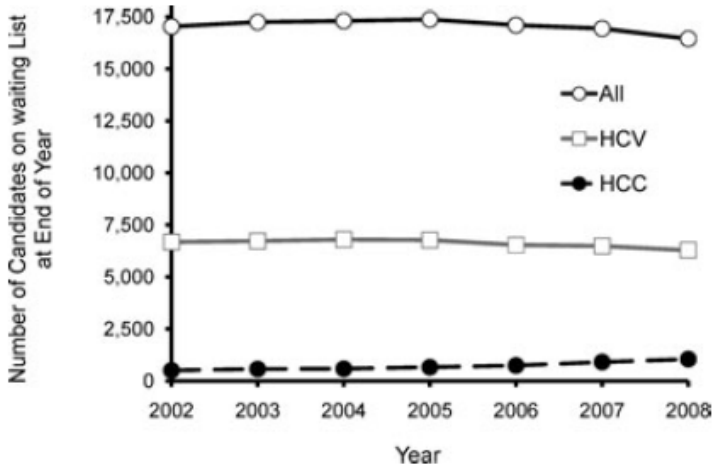
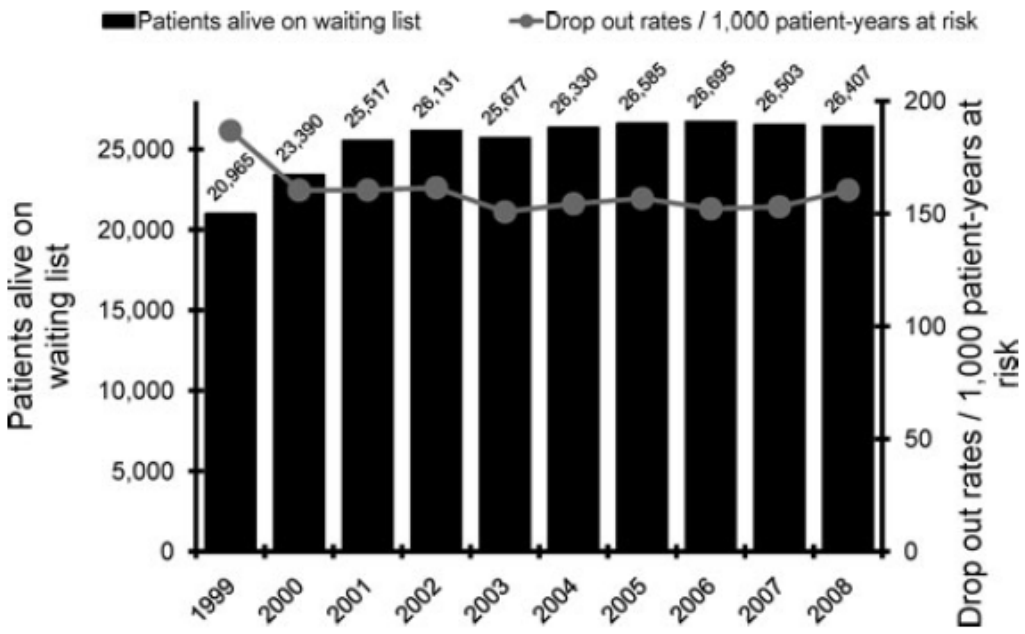


Figure 8. Patients alive on the waiting list at any time during the year and annual drop out (removal due to death or becoming too sick) rates from liver waiting list per 1000 patient-years in the USA [22]



2.53 UK Liver Disease Mortality Statistics

In the last twenty years the number of liver donations has remained fairly constant but the number of people who would benefit from a transplant has increased by an estimated 90% [2]. The number of people requiring a liver transplant therefore far exceeds the number of

liver donations available [2]. As a result of this discrepancy, liver disease is responsible for thousands of deaths in the UK every year (16,087 in 2008) [2]. The 16,087 was a 4.5% increase on 2007 and broke down as 13,805 people in England and Wales, 1,903 in Scotland and 379 in Northern Ireland [7]. Liver disease is the 5th major cause of death in England and Wales after heart disease, cancer, strokes and respiratory diseases [7]. It is also the only major cause of death that still increases annually in the UK as can be seen in Figure 9 [7]. Between 2005 and 2008 liver disease deaths increased in the UK by 12%. 46,244 people died from liver disease in this 3 year period [7]. Liver disease deaths doubled between 1991 and 2008 [7]. The number of liver disease deaths is expected to double again between 2008 and 2028 [7]. Today liver disease kills more people than road deaths and diabetes added together [7]. It is also very worrying that the rest of Europe seems to be learning to take better care of their livers; lowering the rate of liver disease mortalities by reducing alcohol consumption and Hepatitis B and C virus infections [8] [26]. A graph depicting liver disease mortalities for people aged between 45 and 64 years old can be seen in Figure 10 [8].

Figure 9. Graph depicting movements in mortality between 1971 and 2008 in the UK [7]

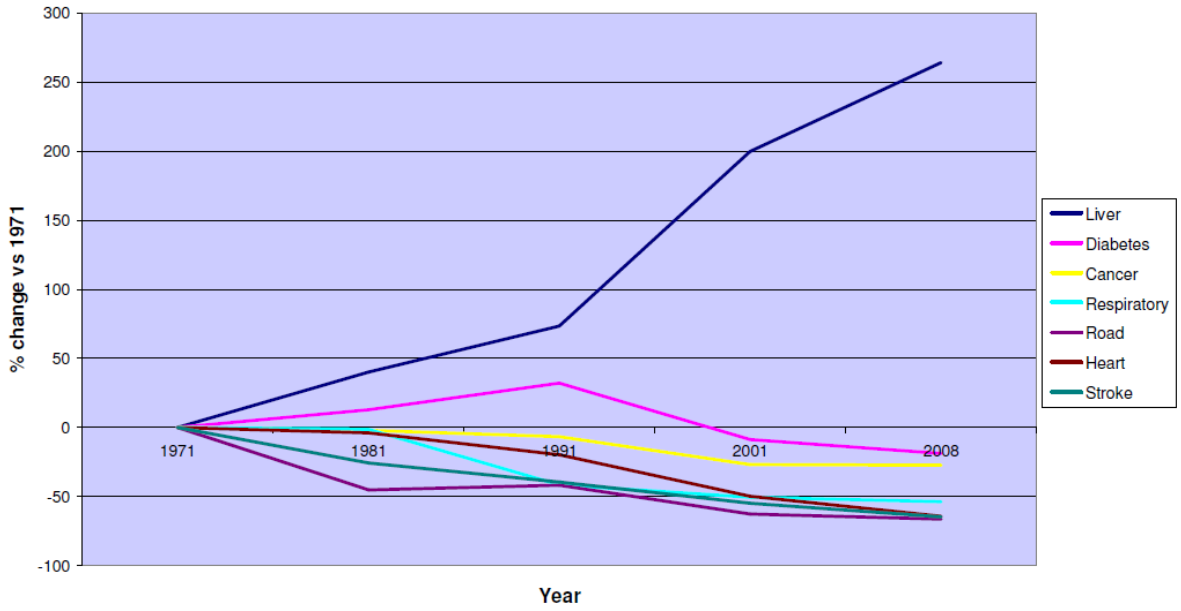
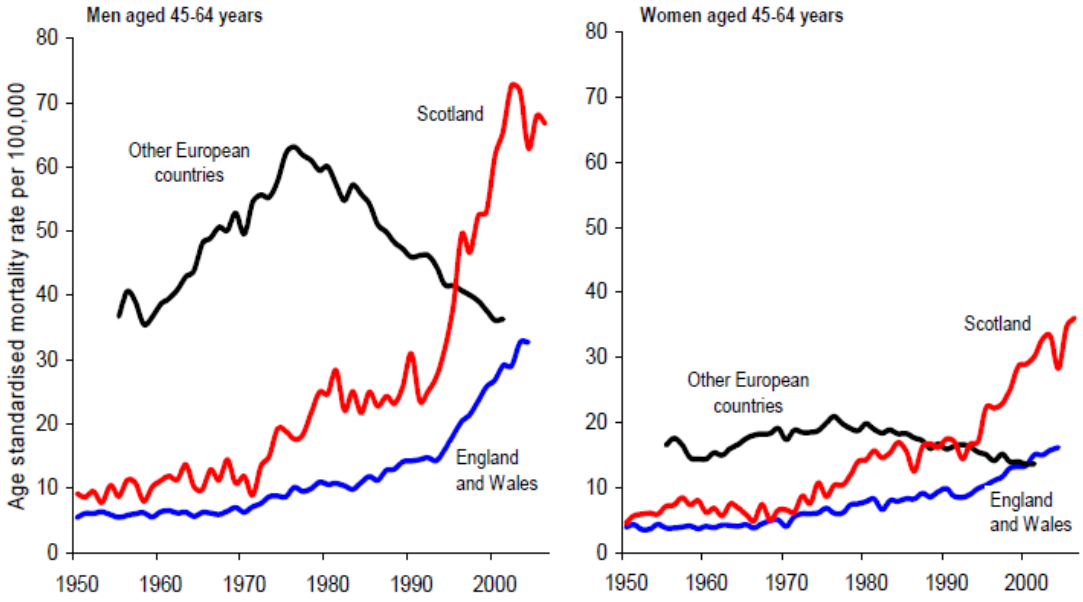


Figure 10. Chronic liver disease and cirrhosis mortality rates per 100,000 population between 1950 and 2006 [8]



3. Alternatives to a conventional liver transplant

3.1 Artificial Liver Device (ALD)

The collection of substances that builds up in the blood during liver failure causes neurological problems, intensifies injury to the liver and other organs, stops residual hepatocytes performing their functions and hinders the liver's regenerative response [27]. This collection includes small molecular weight toxins (like ammonia, free bile acids, phenols and false neurotransmitters), mediators of inflammation (like chemokines, cytokines and anaphylatoxins), vasoactive substances, endotoxin and cell growth inhibitors [27]. ALDs are designed to remove many of the substances known to build up in the blood and cause liver dysfunction, neurological problems, intensify liver injury, reduce the liver's natural ability to regenerate and instigate the failure of the liver, kidneys, immune system or lungs [27]. An ideal ALD would be capable of lowering the levels of toxic substances in the blood as well as providing the lost or impaired liver functions [27]. In simpler terms, an ALD has no biological components and is designed to detoxify a patient's blood [3] [28] [27] [29]. The main cause of hepatic encephalopathy (when liver failure results in a distorted level of consciousness, confusion or even a coma) is believed by some to be hyperammonemia [3] [30] [31] [29]. To filter out the hepatic toxins, ALDs initially used hemoperfusion, hemodiabsorption and hemofiltration [3] [27]. Using sorption therapy to remove toxins from the blood is difficult because the absorptive capacity of chemical adsorbents is limited and unspecified [27]. This is part of the reason why blood detoxification methods like hemofiltration /hemodialysis, charcoal hemoperfusion and more elaborate sorbent based therapies have not been widely used and why when used, they failed to yield any changes in terms of patient survival [27]. These techniques are also linked to side effects including decreasing the white blood cell count and haemorrhaging [3]. Sometimes hormones and growth factors essential to liver regeneration can be filtered out which is obviously another downside of these filtration techniques [3] [27]. To overcome these problems, plasma exchange systems were created [3] [27] [29].

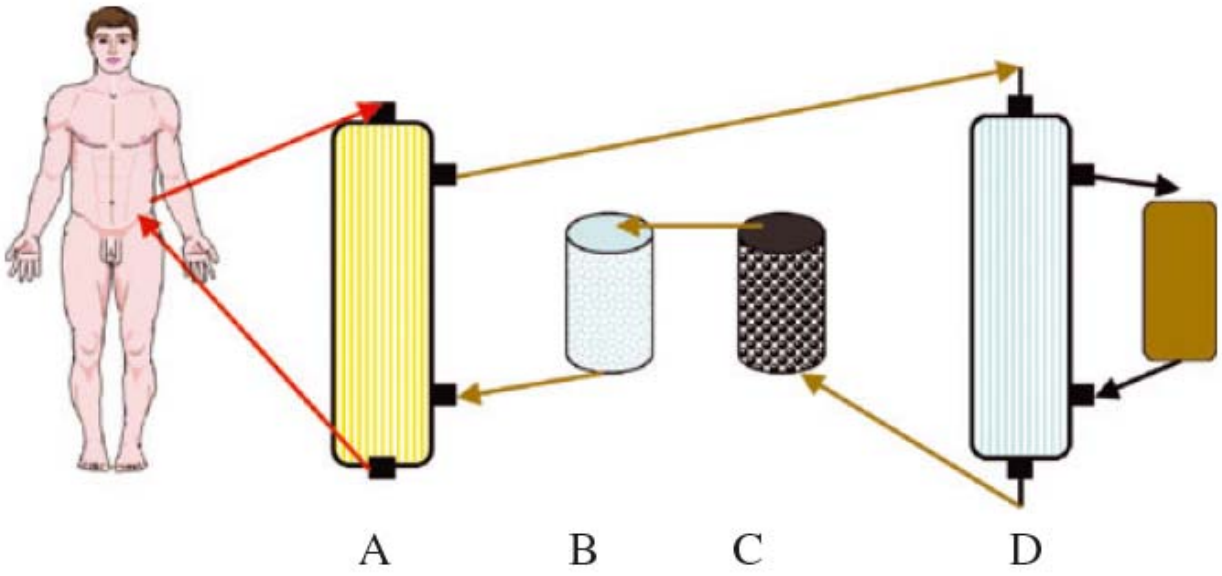
3.11 Plasma Exchange ALD Systems

Plasma exchange systems were designed to allow the toxic substances to be filtered out and the useful factors to be replaced [3] [27] [29]. The progress made in new hollow fibre membranes, blood purification technologies and an improved understanding of the pathophysiology of liver failure led to this type of ALD [27]. These ALDs use either dialysis or exchange mediums (like charcoal or other ion exchange columns) as well as a plasma filter to remove substances [29]. A number of these ALDs including the molecular adsorbent recycling system (known as MARS as seen in Figure 11), Prometheus (Figure 12), single-pass albumin dialysis (SPAD), selective plasma filtration therapy and high performance continuous hemodiafiltration with/without plasma exchange have been clinically trialled [27]. All four of these therapies aimed to provide blood purification [27].

3.12 How the MARS Works

The blood of the patient is guided through the hollow fibre capillaries of the high flux dialyser [32]. The albumin solution circulates in the extracorporeal circuit and passes the membrane counter directionally which allows the albumin bound toxins within the blood to pass through the membrane and combine with the albumin in the MARS circuit [27] [32]. The membrane is permeable to water soluble substances of small and medium molecular weight but impermeable to albumin [27] [32]. The filter then clears the toxins and the albumin is regenerated allowing the acceptance of new toxins the next time the membrane is passed [32]. Continuous veno venous hemodiafiltration (CVVHDF) or continuous veno venous hemodialysis (CVVHD) are used to dialyse the albumin circuit which lessens the load of water soluble toxins [32]. In order for the system to function the MARS requires the albumin solution to be recycled via the charcoal column and anion exchange column [27] [32].

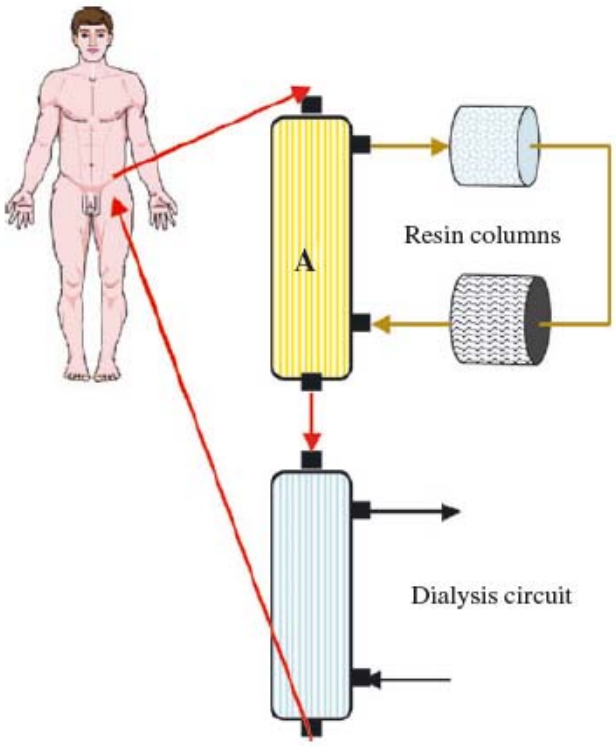
Figure 11. Schematic of the MARS. Blood from the patient is pumped through a high flux dialyser (A) made of the membrane that is impermeable to albumin. Albumin dialysate is recirculated at 250 ml/min through an anion exchange column (B), a charcoal column (C) and a conventional dialyser (D) [27]



3.13 How the Prometheus System Works

The Prometheus system is based on hemodialysis, fractional plasma separation and adsorption [27] [32]. The membrane of this system has a cut off of 250 kDa and is permeable for albumin [27] [32]. The system treats the fractionated plasma with an anion exchanger and neutral resin adsorber [27] [32]. This is then combined with a downstream high flux haemodialysis which removes water soluble substances of low molecular weight [27] [32]. The Prometheus system's 250 kDa membrane is permeable to albumin bound toxins therefore it is believed to be more effective than the MARS in terms of patient detoxification [27] [32].

Figure 12. Schematic of the Prometheus system. Blood from the patient is pumped through a hollow fibre filter which is permeable to albumin (A). Blood ultrafiltrate enters a closed loop circuit containing 2 adsorbent cartridges (resin). Purified albumin re-enters the blood stream and is subjected downstream to high flux haemodialysis [27]



3.14 ALD Summary

None of the 4 systems mentioned have changed the survival of patients but they have improved patients in terms of hepatic encephalopathy and decreasing serum bilirubin [29]. The MARS has been used to stabilise and lower the copper levels of patients with Wilson's disease [29]. A similar outcome has also been achieved using hemofiltration and plasma exchange [29]. Both the MARS and Prometheus systems have demonstrated effective removal of various toxins without triggering any adverse effects but have never consistently increased the survival rates of patients [27] [29] [32]. In summary some ALDs have helped some patients but are yet to yield major increases in terms of patient survival rates [3] [28] [29]. ALDs are expensive and have been linked to hemodynamic problems [3]. A completely artificial device is definitely not the ideal solution for assisting a failing liver [3] [27]. Perhaps an assist device with a biological component might be better. It is worth

noting that most studies evaluating ALDs have used non randomised trials with small sample sizes and no appropriate control so it is hard to draw a true conclusion for ALDs [27] [29].

3.2 Bioartificial Liver (BAL)

Bioartificial Livers are extracorporeal devices that utilise liver cells [3] [27] [29] [32]. Various cell sources have been used in BAL systems including: primary cells (human or animal) or cell lines (tumour or immortalised) [32]. An ideal BAL should be capable of performing all the liver's detoxifying, synthetic and regulatory functions. Today's BALs are incapable of completing all these functions [3] [27] [32]. The majority of human hepatocytes available for Bioartificial Liver Device systems come from human cadaveric livers that are not suitable for transplantation [3] [32]. Primary human cells are biocompatible but isolating enough of them from donor organs for large scale clinical studies would be too complicated [32]. BALs are difficult to seed because human hepatocytes de-differentiate considerably after being plated for a few hours [3]. They lose their hepatic specific gene expression and fail to function correctly [3]. Initially BALs were used to try and bridge the gap before a transplant [3]. BALs do not continue to function properly after a long period of time because as stated above mature hepatocytes scarcely proliferate and quickly lose their hepatic function after being removed from the body [3] [33]. The HepatAssist along with other BAL systems like the ELAD, BLSS, AMC-BAL, RFB, HBAL/TECA-HALSS and MELS have been found safe in phase I clinical trials [34]. A summary of these BALs can be seen in Figure 13 [34]. Only two randomized controlled clinical trials have reported the effectiveness of these BALs [34]. The results reported were disappointing with none of the systems improving the survival rates of patients [34].

Figure 13. Characteristics of certain BAL systems [34]

	Cell source	Bioreactor perfusion	Bioreactor flow rate	MWCO of bioreactor	Oxygenator	Oxygenation gas	Extra detox unit	Barrier filter
ELAD	C3A (400 g), cultured human (400 g), not reported	Before modified→blood Modified→ blood ultrafiltrate	Before modified→ 300-400 mL/min (150-200 mL/min per cartrige) Modified →2 L/min (500 mL/min per cartridge)	Before modified→ 70 kD Modified →120 kD	External	Not reported	No	Before modified→1 µm Modified→0.45 µm
HepatAssist	Porcine (5-7×10^9), cryopreserved	Plasma	400 mL/min	0.15-0.20 µm	External	Not reported	Charcoal column	No
BLSS	Porcine (70-120 g), freshly isolated	Blood	100-250 mL/min	100 kD	External	O_2/CO_2/N_2 mixture	No	No
AMC-BAL	Porcine (1×10^{10}), freshly isolated	Plasma	150 mL/min	Direct plasma-hepatocyte contact	Integral	95% air/ 5% CO_2	No	0.4 µm
MELS	Porcine/human (400-600 g), freshly isolated	Plasma	200-250 mL/min	400 kD	Integral	Not reported	SPAD+ CVVHDF	No
RFB	Porcine (230 g), freshly isolated	Plasma	200-300 mL/min	1 µm	External	95% air/ 5% CO_2	No	0.4 µm
HBAL/ TECA-HALSS	Porcine (1-2×10^{10}), freshly isolated	Plasma	Not reported	100 kD	Not reported	Not reported	Charcoal or bilirubin absorption	No

MWCO: molecular weight cut off; SPAD: single-pass albumin-dialysis; CVVHDF: continuous venovenous hemodiafiltration.

3.21 Monolayers of Hepatocytes in Culture

Primary cultures of hepatocytes fail to proliferate therefore maintaining a stock of them is impossible [35]. Monolayer cultures of hepatocytes also quickly de-differentiate so many of the liver specific characteristics like albumin synthesis and activity of cytochrome-P450-dependent monooxygenases are lost within 48 hours [35]. The cells also fail to maintain their polarity [35]. All of this means that hepatocytes cannot be stored as cultures.

3.22 Cryopreservation of Human Hepatocytes

Human hepatocytes are far from easy to cryopreserve and die when frozen in suspension [36]. There are probably multiple reasons why hepatocytes are so fragile and easy to damage during the freezing procedure [36]. All chemical reactions are slowed down by freezing. The changing of water to ice varies the concentration of the remaining solutes. Cells are damaged during freezing because of the ice crystals and salt concentration [37] [36]. Cells must be frozen without the formation of intracellular ice. In order to minimise freezing injury and ice formation, cells are frozen slowly but rapidly thawed [37] [36]. The cells are also shielded by either a penetrating or non-penetrating cryoprotectant that

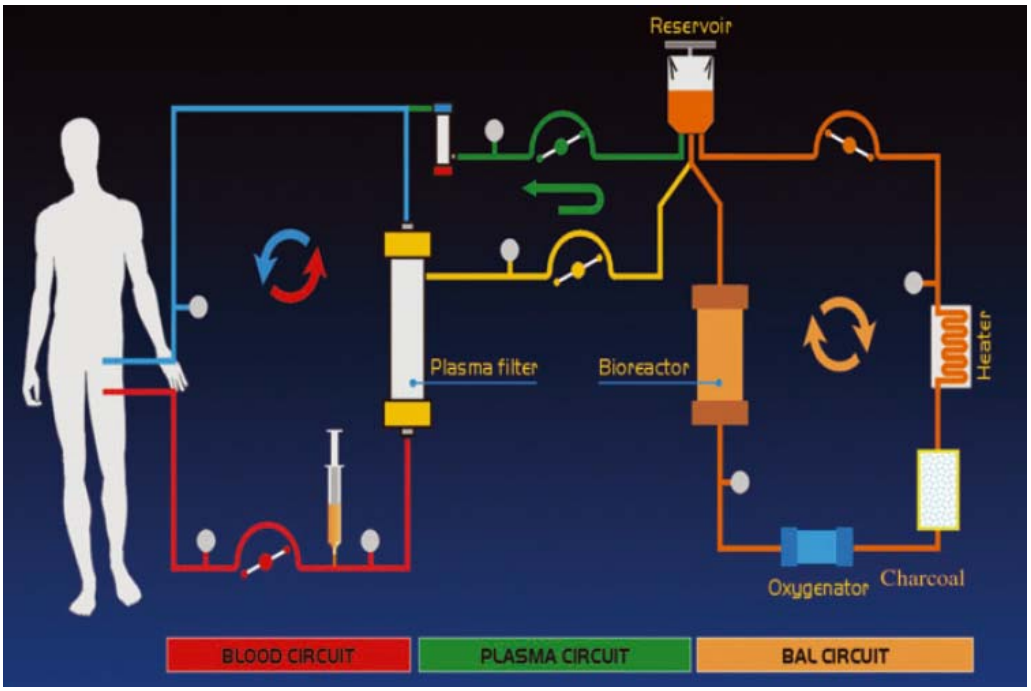
improves post thaw survival [37] [36]. Cryopreserved human hepatocytes can be purchased from Life Technologies but these are for "For research use only. Not intended for human or animal therapeutic or diagnostic use" [38] [39]. They cost from £321.36 per 1.5 ml for 9 to 12 million metabolism qualified, cryopreserved, human hepatocytes [39] to £1,034.12 per 1.5 ml for 4 to 8 million transporter qualified, plateable, cryopreserved human hepatocytes [38]. Looking at the quantities of hepatocytes required by the BALs in Figure 13 makes it obvious that cryopreserved hepatocytes at these prices will never be a financially viable option.

3.23 Porcine Hepatocytes

Porcine tissue is the most used xenograft. Porcine tissue is used because pig organs are a similar size and structure to their human counterparts. When porcine cells are implanted into a human body, an immune response is quickly activated and the tissue is destroyed. To stop this destruction, genetically altered pigs have been proposed as a source [40]. The genetically engineered pigs lacked the gene that created the carbohydrate groups responsible for the quick rejection from the human body.

The HepatAssist (Figure 14) is an example of a BAL that uses porcine hepatocytes. Its hepatocyte bioreactor function is enhanced using a column filled with activated charcoal (which is an adsorbent) [27]. Initially the patient's blood is split into cellular components and plasma using membrane plasmapheresis [27]. The separated plasma is then guided into a high flow plasma recirculation loop. In this loop the plasma passes through the charcoal filter (which removes small molecular weight toxins) and then through an oxygenator (that provides oxygen to the hepatocytes), the heater (which warms the plasma) and finally the hollow fibre bioreactor with 7 billion cryopreserved porcine hepatocytes [27]. After the plasma has been cleaned and processed by the hepatocytes it is brought back together with the cellular components and returned to the patient [27]. The porcine hepatocytes are inserted into the BALs shortly before clinical use [27]. The charcoal column is placed before the bioreactor to try and protect the porcine hepatocytes from the toxic effects of the plasma during liver failure [27].

Figure 14. Schematic of the HepatAssist [27]



The HepaMate system (Figure 15) is the new and improved HepatAssist system. It is also an extracorporeal cell BAL that uses porcine hepatocytes as its biological component [41]. In theory, the HepaMate replaces the whole liver function by combining blood detoxification and liver cell therapy [41]. The company that produces the HepaMate system, HepaLife, has a patented hepatocyte cryopreservation process that is safe and allows the cells to be easily stored and distributed [41]. The system is made up of circuit tubing, a blood plasma separation cartridge, a hollow fibre bioreactor packed with proprietary porcine hepatocytes, an oxygenator, a charcoal column and a plasma reservoir [41]. The HepaMate works by separating the plasma from the whole blood. The plasma is exposed to the BAL and then returned to the patient [41]. The plasma filter separates the plasma from the other whole blood components [41]. The plasma is then treated in the plasma circuit and delivered to the reservoir [41]. The rest of the blood components are recombined with blood from the reservoir that has already been treated [41]. This is returned to the patient [41]. The reservoir sustains a balanced collection of treated plasma (from the bioreactor circuit) and untreated plasma (from the plasma filter) [41]. Treated plasma is returned to the patient through the hemo filter [41]. Initial detoxification of the plasma from the reservoir takes place at the charcoal column [41]. The plasma then flows through the oxygenator to the bioreactor and then back to the reservoir [41]. The

bioreactor has a hollow fibre system that allows plasma to be delivered and is packed with roughly 14 billion porcine hepatocytes [41]. The hepatocytes should eliminate any existing toxins found in the plasma as well as synthesising albumin and other liver specific proteins [41]. These proteins should then be secreted into the patient's blood stream [41]. When all the components have been assembled into the blood/plasma circulation system they are placed on the HepaDrive (Figure 16) [41]. The HepaDrive uses integrated sensors and pumps to control all of the treatment phases and functions [41].

Figure 15. Schematic of the HepaMate [41]

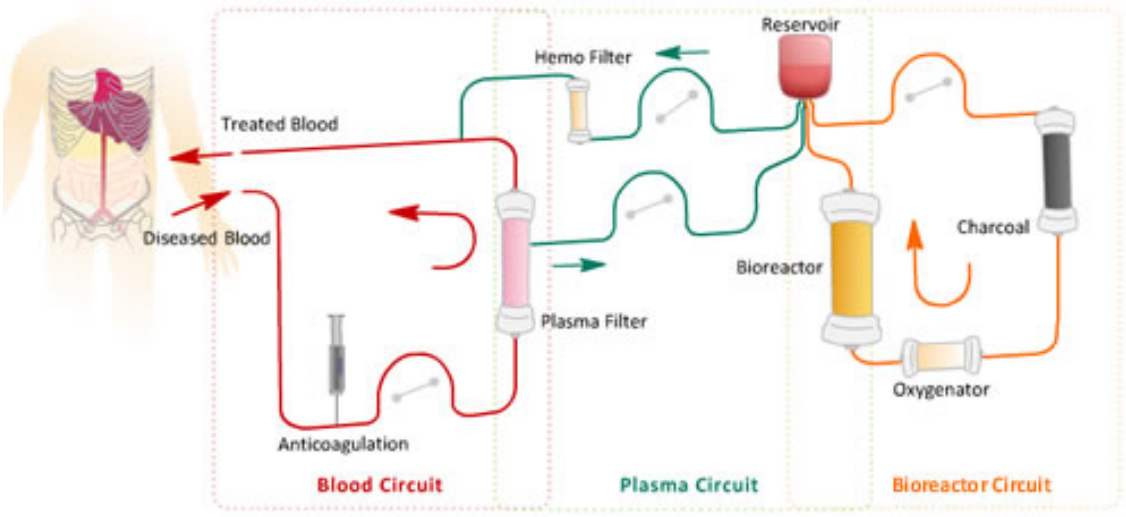
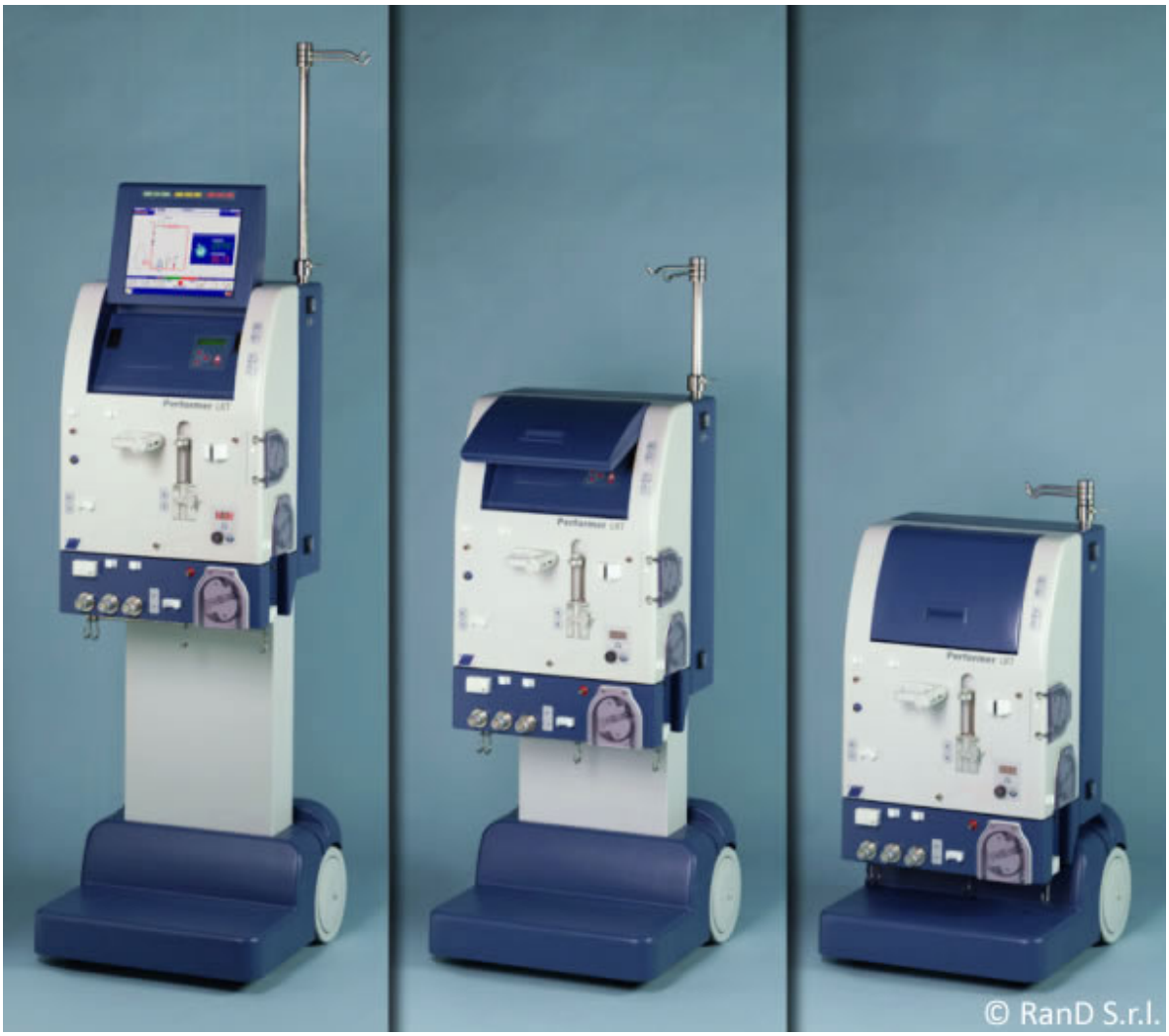


Figure 16. HepaDrive perfusion platform [41]



HepaLife claim that the HepaMate is “the most extensively studied extracorporeal cell-based liver support system” with a total of two clinical trials (Phase I and Phase II/III) involving over 200 patients [41]. They claim that sufferers of acute liver failure displayed survival without a liver transplant and less risk of pre-transplant death. The system helped bridge the gap to transplantation and to recovery (after transplantation) by keeping patients alive and neurologically intact [41]. HepaLife also declared that the HepaMate system improved the survival of drug induced acute liver failure patients [41].

3.24 Other Animal Hepatocytes

It would take the hepatocytes of more than fifty rats to seed a BAL. Sheep are also not used because they can spread a viral infection called Scrapie.

3.25 Hepatoma Cell Lines

Hepatoma cell lines are obtained from cancerous tissue taken from a human liver [4] [36]. Tumour tissue has unregulated cell growth resulting in rapid proliferation in culture. These cell lines are suitable for cryopreservation but have little liver function [42]. Other disadvantages of Hep G2 cells include the release of tumour causing components and extremely low levels of cytochrome P450 [43]. C3A are subclones of Hep G2 cells [43]. The extracorporeal liver assist device (ELAD) has a conventional hollow fibre bioreactor and uses the human cell line C3A [3] [43] [36]. Unlike the Hep G2 cells the C3A cells are reported to show contact inhibition to stop the cells from piling up on one other. Various studies have reported that the ELAD does not improve the patient in terms of survival or biochemical parameters [27] [34].

3.26 Immortalised Hepatocytes

Immortalised Hepatocytes are created by introducing viral DNA into the hepatocyte genome [43]. The viral DNA hinders the hepatocyte's growth regulations resulting in continuous cell proliferation [43]. These cells are also suitable for cryopreservation. The problem with this technique is the cell lines created function erratically. Another downside of this technique is the transfer of oncongenic DNA into a person. Often the simian virus SV40 is used because at its origin of replication there is a deletion in the base sequence that prevents it from replicating [43].

3.3 Hepatocyte Transplantation

Hepatocyte transplantation has also been investigated as a method for improving liver function in patients suffering with liver failure [44] [45]. Studies have indicated that infused hepatocytes can function in a patient for nine months [44] [45]. A hepatocyte infusion of around 20% of the normal hepatocyte mass is required to bridge the gap to transplantation [44]. As well as the previously stated problems with hepatocytes like liver donor shortages, they are difficult to cryopreserve and rapidly lose their hepatic function ex vivo; hepatocyte transplantation also has the problem of managing immunosuppression. This is problematic because in many cases it is difficult to detect early rejection of the cells [45]. Immunosuppression would also be a serious problem if transplanting animal hepatocytes like porcine hepatocytes. In 2011, doctors in London implanted donor liver cells into a 2 month old who was close to death [46] [47]. His liver was failing due to a herpes simplex virus [46] [47]. The injected liver cells processed toxic substances and generated vital proteins [46]. The cells functioned like a temporary liver and allowed the boy's own liver to recover [46] [47]. The liver cells were coated in a chemical present in algae [46]. This coating stopped the cells from being rejected and destroyed by the immune system [46]. A massive advantage of this technique would be that patients do not need immunosuppressants. A large clinical trial is required to check how successful this technique could be on a wider scale.

3.4 Cell Source Summary

Primary cultures of hepatocytes fail to proliferate therefore keeping a stock of them is impossible. Cryopreserving hepatocytes is difficult and cryopreserved human hepatocytes are too expensive and are not available in a large enough supply. There has been success using pig hepatocytes in BALs but these are not a viable option due to the fear of spreading viral diseases [3] [27] [43]. A study using in vitro experiments proved that BALs using porcine hepatocytes were capable of infecting primary human hepatocytes with the porcine endogenous retrovirus (PERV) [48] [27] [43]. Using porcine hepatocytes in BAL devices is even illegal in some European countries [48]. Pig hepatocytes are also incapable of completing sulphation reactions. These reactions are essential for managing the active concentration of hormones and detoxifying chemicals in the human body. Currently

available hepatoma and immortalised cell lines demonstrate only a small percentage of the metabolic activity displayed by primary human liver cells [32]. Therefore it would take a huge cell mass for a cell line BAL to be successful [32]. The cells are separated from the patient's blood stream but there is still a risk of metastasis formation [32]. It looks like BALs and hepatocyte transplantations require a fully functional, highly differentiated human hepatocyte line in order to treat or manage liver disease [3] [32] [34]. The source of these cells is problematic and recently the possibility of hepatocytes derived from stem cells have been proposed as an answer.

4. Stem Cells

Stem cells are derived from adult (somatic) and embryonic sources. They have tremendous therapeutic potential but much more research is required before they are used in standard clinical applications. Stem cells are a promising cell source for cell therapies and tissue engineering bone, cartilage, ligaments, tendons and muscle. Stem cells can renew themselves through cell division, be induced into many different cell types, engraft within various tissues and exert beneficial effects on other cell types. When a stem cell divides, each of the two resulting cells has the potential to either remain as a stem cell or differentiate into a more specialised cell (like an erythrocyte or neuron) [49]. There are two main differences between stem cells and other cell types; stem cells are unspecialised cells that can renew themselves through cell division (even after long periods of inactivity) and under certain conditions, stem cells can be induced to differentiate into organ or tissue specific cells [49]. In the adult body, stem cells take part in tissue maintenance and repair. Stem cells routinely divide in the gut to repair and restore damaged tissues but in the heart, they only divide under particular conditions [49].

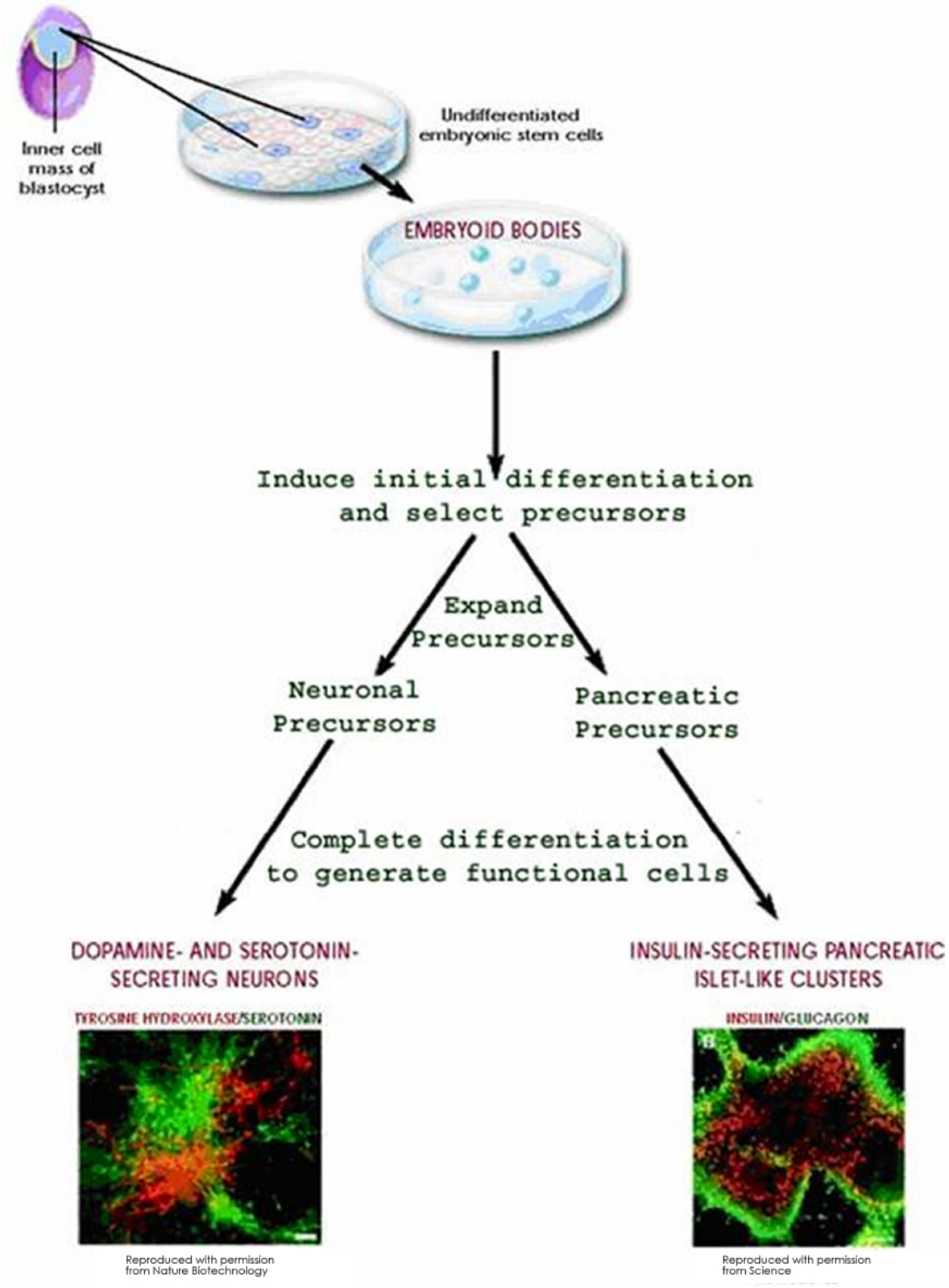
4.1 Embryonic Stem Cells

Embryonic stem cells (ESCs) are derived from embryos. ESCs do not come from eggs fertilised in a woman's body, most are donated (with the donors permission) by in vitro fertilisation clinics [50]. In 1981, embryonic cells were derived from mice and in 1998 the first stem cells from human embryos were grown and isolated [49] [51]. Considering that was only fourteen years ago, this makes the progress made in embryonic stem cells even more astonishing. The embryos used in ESC studies were originally fashioned through in vitro fertilisation for reproductive purposes. When no longer required for that purpose, they were donated to research with the donor's consent. Human ESCs are created by transferring cells from an embryo (that has yet to be implanted into the uterus wall) into a culture dish containing a nutrient broth (culture medium) [50]. The cells proliferate over the surface of the culture dish [50]. The inner surface of the dish is usually coated with mouse embryonic skin cells that do not divide; this is known as a feeder layer [50]. These mouse cells at the bottom of the dish provide the embryo cells with an adhesive surface to

which they can attach [50]. The feeder cells also release nutrients into the culture medium [50]. ESCs can now be grown without mouse feeder cells; this method eliminates the risk of viruses or other macromolecules being transmitted from the mouse cells to the human cells [50]. If the plated cells survive and proliferate enough to crowd the dish then they are removed and re-plated into multiple fresh dishes [50]. Re-plating the cells is replicated many times over many months [50]. After the cell line is set up, the initial cells yield millions of ESCs [50]. In cell culture, the ESCs that have proliferated but not differentiated for a prolonged period of time are pluripotent [50]. Batches of cells can be frozen and transported to other laboratories at any point in the process [50].

ESCs have to be grown in culture under appropriate conditions to keep them undifferentiated (unspecialised) because if the cells clump together and form embryoid bodies, they will start to differentiate spontaneously [50]. Controlling the differentiation of ESCs is essential in order to produce cultures of specific cell types like hepatocytes [50]. Differentiating ESCs into specific cells can be controlled by varying the culture medium's chemical composition, altering the cells by inserting specific genes or changing the culture dish's surface [50]. Two protocols for directing differentiation of ESCs into specific cell types are shown in Figure 17 [50].

Figure 17. Two protocols for directed differentiation of ESCs into specific cell types [50]



Human ESCs seem to have the ability to divide indefinitely in vitro as well as the capability to differentiate into any somatic cell type [52]. Several studies have shown human ESCs differentiating into various cell types including: neural cell types (neurons, glia and oligodendrocytes), osteoblasts, cardiomyocytes, haemopoietic progenitors, β cells and most importantly for this study, hepatocytes [52]. In order to decrease the chance of spontaneous teratoma formation, the cells need to be differentiated to a high purity endpoint and injected in small cell volumes [51]. There are ethical concerns and objections associated with using ESCs for therapeutic cloning.

4.2 Somatic Cell Nuclear Transfer (SCNT)

Studies have shown that somatic cell nuclear transfer (SCNT) techniques can be used on human oocytes and result in blastocyst formation from which embryonic stem cell can be derived [52]. Autologous ESCs for therapeutic purposes could potentially be created using SCNT but this remains ethically complex and controversial [52]. There is also a worry that nuclear transfer human embryonic stem cells could cause the formation of teratomas after in vivo implantation [52]. Nuclear transfer ESC lines are similar to human ESC lines in terms of their differentiation potential [52]. If SCNT stem cells were to be used clinically, a cell line for each individual patient would need to be produced [52]. However a bank of human ESCs could provide suitable tissues for most patients [52].

4.3 Foetal Stem Cells

There are many stem cell populations present in the foetus as it develops however their potential use in clinical applications has hardly been explored [52]. This is because of the obvious ethical issues of using cells from foetuses and the risks associated with intrauterine procedures [52]. A less controversial option could be to use the circulating human foetal mesenchymal stem cells as a source of cells for therapy [52]. These cells are only found circulating in the first trimester and closely resemble the haemopoietic populations found in foetal bone marrow and liver [52]. Studies have shown that these stem cells engraft into

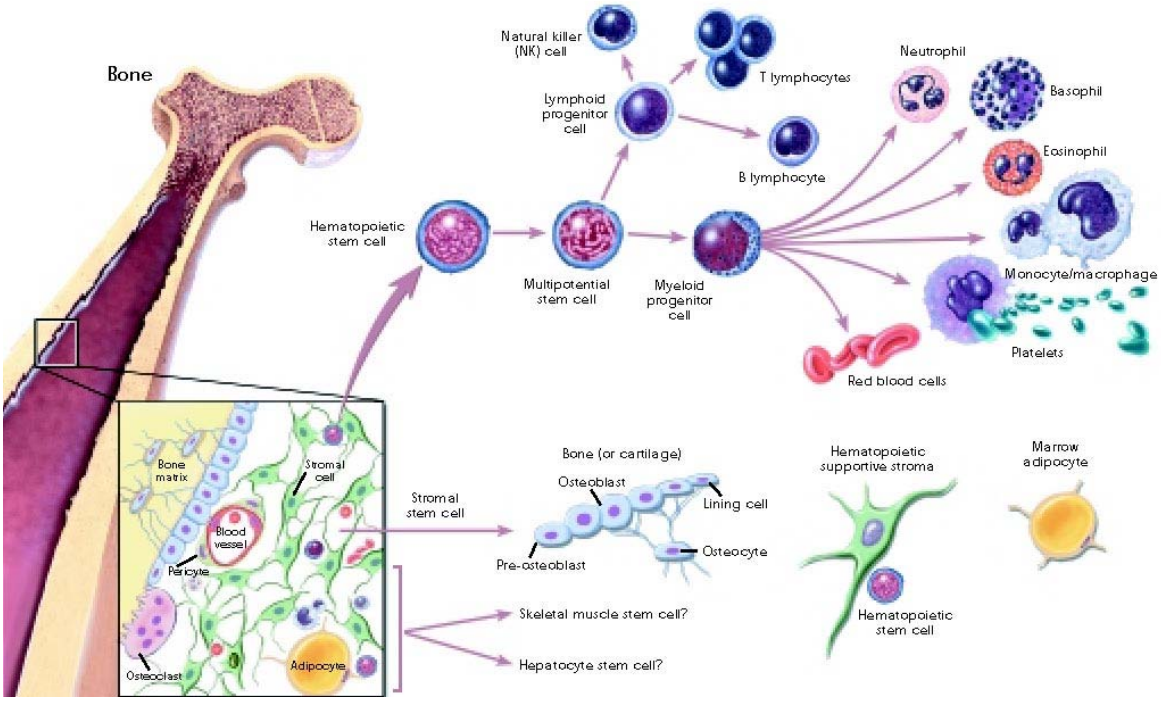
many organs and undertake specific tissue differentiation when transplanted a sheep model [52].

4.4 Adult Stem cells

Adult stem cells or somatic stem cells are thought to be undifferentiated cells that are found beside differentiated cells in an organ or tissue. They can proliferate and divide to produce some or all of the main specialised cells of that organ or tissue. They primarily maintain and restore the tissue in which they are found. Adult stem cell transplants have actually existed for decades in the form of bone marrow transplants. Adult stem cells have been found in many organs and tissues including: the brain, skin, bone marrow, blood vessels, peripheral blood, skeletal muscle, gut, heart, liver and even the teeth [53]. It is believed that the cells reside in a particular area of tissue called the "stem cell niche" [53]. In each tissue, there are usually only a very small number of stem cells and their ability to divide is limited ex vivo. This is why generating large quantities of these stem cells is so difficult. Scientists are continually attempting to find better ways to grow large amounts of adult stem cells [53]. Those cells will then have to be manipulated into generating specific cell types [53].

Figure 18 shows some in vitro and in vivo examples of differentiating adult stem cells. Mesenchymal stem cells produce a selection of cell types including: osteocytes, chondrocytes and adipocytes. Hematopoietic stem cells generate all blood cell types including: erythrocytes, macrophages, lymphocytes, natural killer cells, monocytes, neutrophils, eosinophils and basophils. Neural stem cells in the brain make three major cell types: neurons, astrocytes and oligodendrocytes. In the skin, epidermal stem cells formulate keratinocytes. Epithelial stem cells in the digestive tract create multiple cell types including: goblet cells, absorptive cells, enteroendocrine cells and paneth cells [53]. Some adult stem cells like neural stem cells are hard to acquire from a living donor [52]. Other problems associated with adult stem cells include their declining abundance and potency with age or possibly disease [52]. Several experiments have reported that some adult stem cell types can differentiate into cell types found in other organs like neural stem cells differentiating into blood cells [53]. This is known as transdifferentiation, whether this can actually occur in humans is under scientific debate [53].

Figure 18. Hematopoietic and stromal stem cell differentiation [53]



4.5 Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are derived from somatic cells and are genetically reprogrammed to assume an embryonic stem cell like state. iPSCs were initially reported in mice in 2006 and then in humans in 2007 [51] [54]. Unlike ESCs, iPSCs would not require immunosuppressive drugs because the cells are autologous so they would not be rejected [51]. iPSCs could have a massive effect on the future of medicine with the potential to help understand cell differentiation or dedifferentiation, establish individualised iPSCs for medical applications without having to harvest allogeneic embryonic stem cells or deal with nuclear transfer and produce patient specific iPSCs to examine genetic background and how diseases affect patients [55]. The somatic cells are reprogrammed into pluripotency using defined factors instead of the large number of nuclear and cytoplasmic factors in the oocyte, as in a nuclear transfer [55]. Studies have shown that ectopic expression of four genes (Oct-4, Sox2, Klf4, and c-Myc) is adequate to reprogramme human fibroblasts into induced pluripotent stem cells [55] [56] [57] [58]. Human endometrial fibroblasts (taken from donors in their 30s or 40s) can also be reprogrammed to produce pluripotent stem

cell lines by the retroviral transduction of Oct-4, Sox2, Klf4, and c-Myc (OSKM) in female donors [56]. Completely reprogrammed iPSCs exhibit various properties similar to those of ESCs [56] [57] [58]. iPSCs are morphologically similar to ESCs, they can also self renew and differentiate into three germ layers like ESCs [56] [57] [54]. iPSCs are also indistinguishable from ESCs in terms of gene expression, telomerase activity and surface antigens [56] [57]. Currently, not a lot is known about the exact reprogramming mechanisms that allow a somatic cell to be reversed into a pluripotent state [55]. Studies into the intracellular mechanisms of the reprogramming process are required [55]. Although iPSCs avoid a lot of the ethical issues associated with embryonic or foetal stem cells, there are ongoing concerns about the distribution of subject specific genome sequence data and because iPSCs and iPSC derived cells cannot be tested in humans, animal testing is required to assess pluripotency and therapeutic potential [57]. There is no doubt that iPSCs have the potential to offer advantages in clinical applications but several obstacles must be overcome first including: does using viral vectors (some are oncogenes) increase the risk of tumour formation, differentiating iPSCs into the functional cells required has yet to be successful in vivo and acquiring pure populations of target cells remains difficult.

4.6 Mesenchymal Stem Cells (MSCs)

There are a large number of potential sources of MSCs including: adipose tissue, olfactory mucosa, bone marrow, articular cartilage, muscle and other tissues [59] [60]. Autologous adipose stem cells (ASCs) have received substantial attention as a cell source in bone and cartilage repair [59]. Adipose tissue is regarded as a good source of stem cells for clinical use because of the ease with which it can be retrieved [59]. The process of retrieval is minimally invasive and suitable for outpatient clinics [59]. Another advantage is the large number of cells retrieved (up to 25,000 adherent ASCs per gram) [59]. Mesenchymal stem cells can be purified and propagated in vitro in order to generate osteoblasts, chondrocytes and adipocytes [52]. The cells generated are dependent on the growth factors used to stimulate the MSCs [52]. There are obvious advantages to using autologous mesenchymal stem cells in clinical applications because there would be no issue of rejection.

4.7 Stem Cell Summary

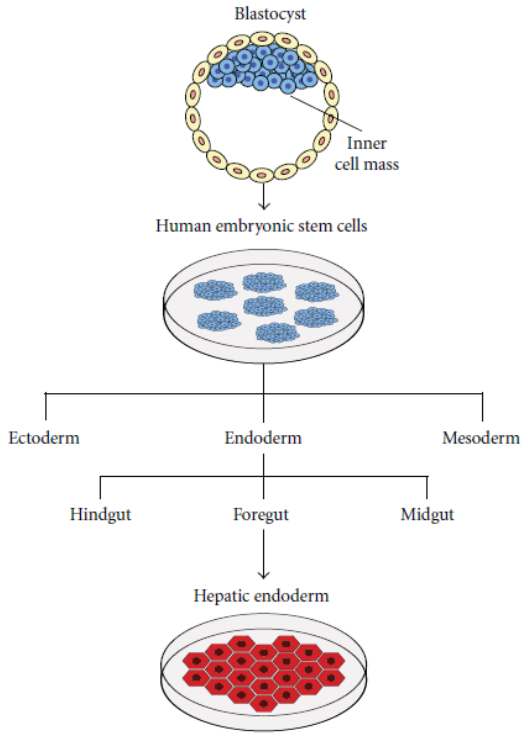
If stem cells can be reliably and safely differentiated they could be used to treat not only liver disease but also other diseases like Parkinson's disease, traumatic spinal cord injury, heart disease, Duchenne's muscular dystrophy, diabetes as well as loss of vision and hearing [50]. Before stem cells can be used clinically they must proven stable, incapable of transmitting genetic mutations or harmful pathogens and unable to form unwanted tissues or teratocarcinomas [52].

5. ESC Derived Hepatocytes

5.1 Introduction

A simple culture system that efficiently differentiates embryonic stem cells into hepatocytes is essential in providing a cell source for clinical applications. When compared with foetal and adult bi/multipotent stem/progenitor cells, pluripotent embryonic stem cells have unique pluripotent versatility [61]. The cell types they can form are unrestricted and include neurons, cardiomyocytes and hepatocytes [61]. Embryonic stem cells have the potential to differentiate into hepatocytes both in vivo and in vitro [62]. Human embryonic stem cells can be programmed into functional hepatic endoderm by sending development signals [3]. Growth factors (usually proteins or steroid hormones) are naturally occurring substances that bind to receptors on the cell surface and can induce cellular growth, proliferation and differentiation. Hepatic differentiation can be induced from embryonic stem cells by adding various growth factors including hepatocyte growth factor (HGF), oncostatin M (OSM), acidic fibroblast growth factor (aFGF), basic FGF (bFGF) and the steroid dexamethasone (DEX) [63]. One way of achieving hepatic differentiation is by spontaneous differentiation. Spontaneous differentiation of human ESCs leads to the formation of embryoid bodies made up of a diverse cell population of the three germ layers: ectoderm, mesoderm and endoderm [3]. The three germ layers interact to develop all the tissues and organs that make up a developing embryo. The embryoid bodies have been observed spontaneously differentiating into hepatic endoderm with limited efficiency [3]. After embryoid body formation, specific growth factors have been used to drive hepatic differentiation [3]. The resulting differentiated cells then require purification [3]. Embryoid bodies derived from embryonic stem cells can differentiate into mature hepatocytes both in vivo and in vitro [62]. Another way of attaining hepatic endoderm is by direct (or directed) differentiation, this technique uses an inducer [62]. Directed differentiation is shown in Figure 19 [3]. Many believe direct differentiation without the formation of embryoid bodies is a faster and more efficient way of producing hepatic endoderm [3]. Usually the in vitro techniques involve both spontaneous differentiation (with the formation of embryonic bodies) and directed differentiation [62].

Figure 19. Schematic diagram of the derivation of hepatic endoderm from human ESCs [3]



5.2 The Importance of Growth Factors when Deriving Hepatocytes from ESCs

Activin A is a member of the transforming growth factor beta (TGF-β) superfamily. Activin A has been shown to differentiate embryonic stem cells into hepatic endoderm cells [64] [65] [62] [66] [67] [68] [69]. Activin A inhibits DNA synthesis, induces cytoskeleton reorganisation and stimulates extracellular matrix production (to regulate the structure of the liver) [67]. Yu et al demonstrated the generation of hepatic endoderm cells by adding activin A for five days to one day old embryoid bodies formed from human embryonic cells [70]. The cells were then co-cultured with mitomycin (a chemotherapy drug) treated 3T3-J2 (mouse fibroblasts) feeder cells [70]. After being co-cultured, the hepatic endoderm cells produced hepatocyte like cells [70]. These cells had the proliferation potential to be cultured for a period of more than 30 days [70]. After being extensively expanded, the cells co-expressed the hepatic marker α-fetoprotein (AFP) and albumin [70]. This confirmed the cells were like hepatocytes. A combination of activin A and another multifunctional

cytokine of the TGF-β superfamily, bone morphogenic protein (BMP), has also been used to differentiate embryonic stem cells into hepatocyte like cells [67]. However recent work has indicated that complementing activin A with the signalling protein, Wnt3a, improves the function of developing hepatic endoderm [71]. This discovery led Hay et al to the first instance of highly efficient and expandable derivation of functional hepatic endoderm (from human embryonic stem cells) [71]. Activin A and Wnt3a have been used to derive cells from ESCs that demonstrate the metabolic function and phenotype of mature human hepatocytes [71]. Without their specialised tissue microenvironment, stem cells usually have limited function [3]. Essential in vivo components and interactions that make the microenvironment "niche" include cell to cell contacts, growth factors as well as cell matrix adhesions which regulate the production, maintenance and repair of tissue [3].

Activin A, BMP and Wnt3A are not the only growth factors that can promote differentiation of ESCs into hepatic cells. Growth factors and hormones like fibroblast growth factor (FGF), hepatocyte growth factor (HGF), OSM (oncostatin M) and DEX (dexamethasone) have been used to induce hepatic differentiation in embryoid bodies from embryonic stem cells since 2001, when Hamazaki et al demonstrated their ability [72]. As the name suggests, HGF plays an important part in hepatic differentiation [62]. HGF is a growth factor that induces hepatocyte mitosis as well as promoting hepatic differentiation, acquisition of hepatocyte like phenotype and liver specific functionality [67]. The FGF family of growth factors can induce the definitive endoderm to differentiate into hepatic cell lineage [67]. Epidermal growth factor receptor (EGF) augments liver regeneration, inhibits cellular death and promotes hepatocyte architecture, feasibility and functionality [67]. OSM and DEX are useful because of their excellent reprogramming capacity; they also work well when combined with EGF to promote liver like differentiation [67]. Combining activin A with HGF, DEX and basic fibrous growth factor (bFGF) has been used as a technique to obtain functional hepatocytes from mouse embryonic stem cells [62].

Serum replacement dimethylsulfoxide (SR/DMSO) has been applied directly to human ESCs to induce differentiation. The resulting hepatocytes made up approximately 10% of the cells produced, demonstrated typical polygonal morphology and performed multiple hepatocyte functions [73]. The low yield of hepatocytes indicated that DMSO induced differentiation is not lineage specific [73]. Lakshmi et al reported that sodium butyrate contributes to a more homogeneous hepatocyte differentiation [74]. Using sodium

butyrate and activin A together has been found to induce definitive endoderm differentiation from human ESCs [75] [76]. Hay et al demonstrated that a significant number of the cells in sodium butyrate and activin A combined treatment displayed early hepatocyte morphology, whilst cells treated with only activin A resulted in less hepatocyte formation and there was no clear hepatocyte differentiation in sodium butyrate treatment alone [76].

5.3 The Importance of a Stepwise Procedure when Deriving Hepatocytes from ESCs

Differentiating mature hepatocytes from human embryonic stem cells is inefficient because of the low yield and considerable cellular heterogeneity [62]. Using a stepwise protocol that first includes the generation of definitive endoderm from human embryonic stem cells and ends with mature hepatocytes has led to a more efficient production of hepatocytes with higher purity (around 70%) [64] [62] [77] [66] [71] [68] [77] [78] [79] [80].

In 2010, Touboul et al defined chemical conditions that did not use any feeder cells or serum [68]. The human embryonic stem cells were initially differentiated into homogenous endoderm cells from which mature hepatocytes can be efficiently generated [68]. The differentiated hepatic cells were recognised and characterised by analysing the expression patterns of proteins and genes including: albumin (ALB), hepatocyte nuclear factor 3β (HNF3β), HNF4α, cytochrome P450 enzymes, tyrosine aminotransferase (TAT) and tryptophan-2, 3-dioxygenase (TO) [68].

In 2011, Bukong et al used a novel approach that demonstrated human embryonic stem cell differentiation into functional and morphological yet immature hepatocytes [67]. This approach used recombinant growth factors and metabolites sequentially to induce the differentiation of human ESCs to hepatocytes. Initially the embryonic stem cell H1 line was subcultured on a feeder layer [67]. The human ESCs were induced into endodermal differentiation [67]. The human embryonic stem cells were differentiated using the sequential deployment of growth and differentiation factors between three and seven days after the last subculture [67]. Throughout differentiation the culture medium was enhanced with serum as follows: non heat inactivated foetal bovine serum 0.5% daily

(throughout stage 1), upped by 1% daily to 5% (throughout stage 2) and upped by 1% daily to 10% (throughout stage 3) [67]. During stages 2 and 3, the culture media was also complemented with 0.1% insulin-transferrin-selenium (ITS) [67]. The next step involved early/late hepatic specification [67]. The final step was hepatocyte maturation (shown in Figure 20), this required stepwise combinations of activin A and fibroblast growth factor 2 (FGF2) everyday for seven days, succeeded by bone morphogenic protein 2 (BMP2) and FGF4 everyday for seven days. The final stage of hepatocyte maturation involved adding hepatocyte growth factor 4 (HGF4), FGF4, FGF10 and epidermal growth factor (EGF) everyday for fourteen days [67]. Throughout differentiation, specific inhibitors and stimulators were added sequentially [67]. LY294 002 hydrochloride is a specific inhibitor of phosphatidylinositol 3-kinases (which are involved in cancer). Retinoic acid (RA) was used to promote the metabolic activity of the differentiated hepatocytes [67]. RA was also used to protect the hepatocytes from oxidative stress and aid in their differentiation [67]. SB431542 hydrate was used to help promote differentiation [67].

Figure 20. Human embryonic stem cell differentiation using a novel three step process under chemically defined conditions. The measurements in ng/ml and μM refer to the daily doses added [67]

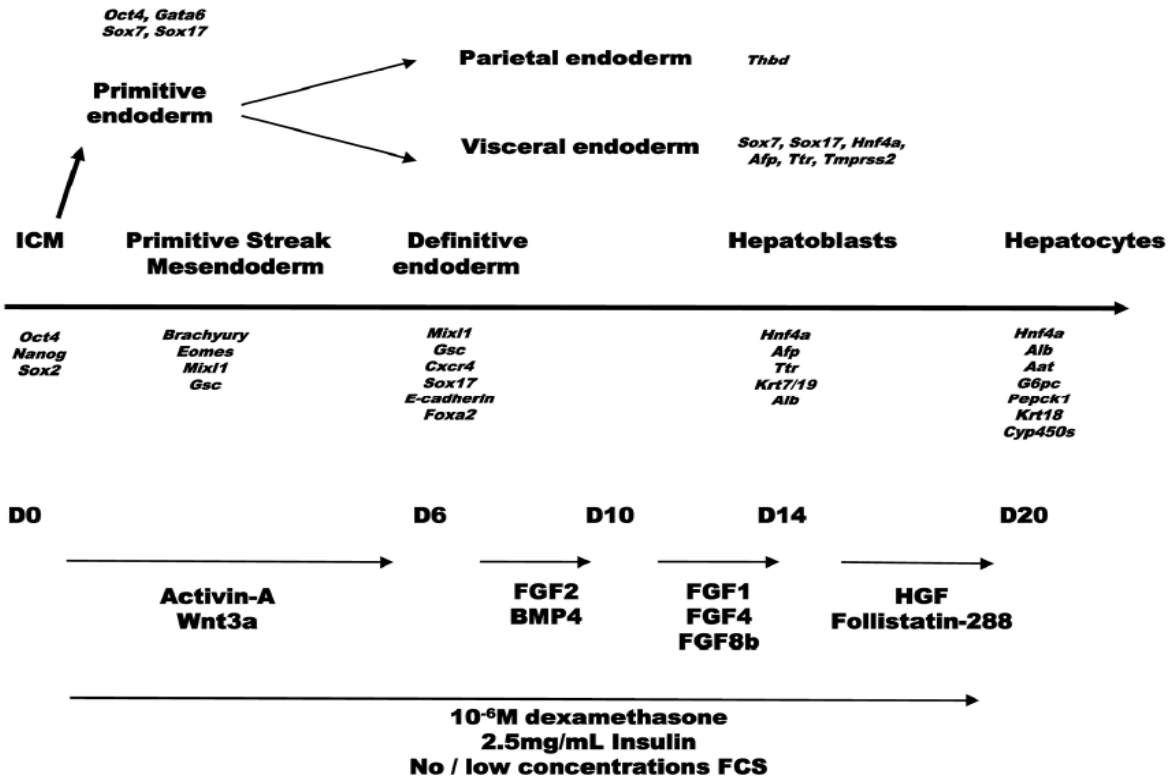
Duration (days)	Differentiation Stage	Chemical condition	
3–7 days post-passage	Undifferentiated	hESC media	
7 days (Stage I)	Endodermal differentiation	+Activin A (10 ng/ml) +FGF-2 (50 ng/ml)	
7 days (Stage II)	Hepatic specification	+FGF-4 (20 ng/ml) +BMP-2 (10 ng/ml) +LY294 002 hydrochloride (25 ng/ml)	
14 days (Stage III)	Hepatic maturation	Entire duration of stage III	+FGF-10 (50 ng/ml) +RA (10 ng/ml) +FGF-4 (25 ng/ml) +HGF (0.005 U/ml) +EGF (20 ng/ml) +SB431542 hydrate (1 ng/ml)
		Added during last 2 days of stage III	+Osm (5 ng/ml) +Dex (0.05 μM)

Viable cells with hepatocyte morphology and shape were produced using this protocol [67]. Gradual changes were observed in cell transcriptome [67]. These changes included: the upregulation of differentiation promoting HNF4, POU5F1, GATA4 and GATA6 transcription factors, low levels of Nanog and steady levels of stemness promoting SOX2 [67]. The derived hepatocytes expressed CD81, keratin 8, low density lipoprotein (LDL) receptor and alpha-antitrypsin [67]. The levels of proliferation marker Ki-67 and α-fetoprotein in the derived hepatocytes remained elevated [67]. The derived hepatocytes

performed functions that stem cells do not including: albumin production, alanine aminotransferase, LDL uptake and they also had functional alcohol dehydrogenase [67].

Protocols that replicate mammalian liver development have been developed to differentiate embryonic stem cells with pluripotent characteristics into hepatocyte like cells. Roelandt et al developed a protocol for human ESCs that makes use of cytokine cocktails to replicate the four major stages of embryonic and foetal liver development [81]. The ESCs are differentiated into cells with characteristics of primitive streak/mesendoderm /definitive endoderm, hepatoblasts, then finally into cells with the same phenotypic and functional characteristics of hepatocytes (as seen in Figure 21) [81]. Pluripotent cells within the inner cell mass (ICM) are fated to either primitive endoderm or epiblasts. Primitive endoderm gives rise to visceral endoderm and parietal endoderm. During gastrulation, epiblast cells ingress through the primitive streak to form mesendoderm and then definitive endoderm. Definitive endoderm cells are fated to populate the endodermal organs like the liver, gut and pancreas. The first stage of this four stage technique involved replicating gastrulation (when one germ layer, blastula, becomes three germ layers, gastrula) by exposing the cells to 100 ng/ml of activin A and 50 ng/ml of Wnt3a [81]. In stage two, 50 ng/ml of BMP4 (secreted in vivo by the adjacent septum transversum mesenchyme) and 10 ng/ml of FGF2 (secreted in vivo by the adjacent cardiac mesoderm) were added to stimulate definitive endoderm to a hepatic fate [81]. Stage three induced proliferation and initial maturation of the early hepatoblasts and involved 50 ng/ml of FGF1, 25 ng/ml of FGF8b and 10 ng/ml of FGF4 [81]. The purpose of stage four was to induce a mature hepatocyte phenotype. This stage consisted of 20 ng/ml of HGF (general hepatotrophic cytokine) and 100 ng/ml of Follistatin-288 [81]. Follistatin-288 was used to stimulate hepatic rather than cholangiocyte differentiation [81]. 1 mM of DEX was also added to induce expression of mature hepatic specific genes as well as 2.5 mg/ml of insulin [81].

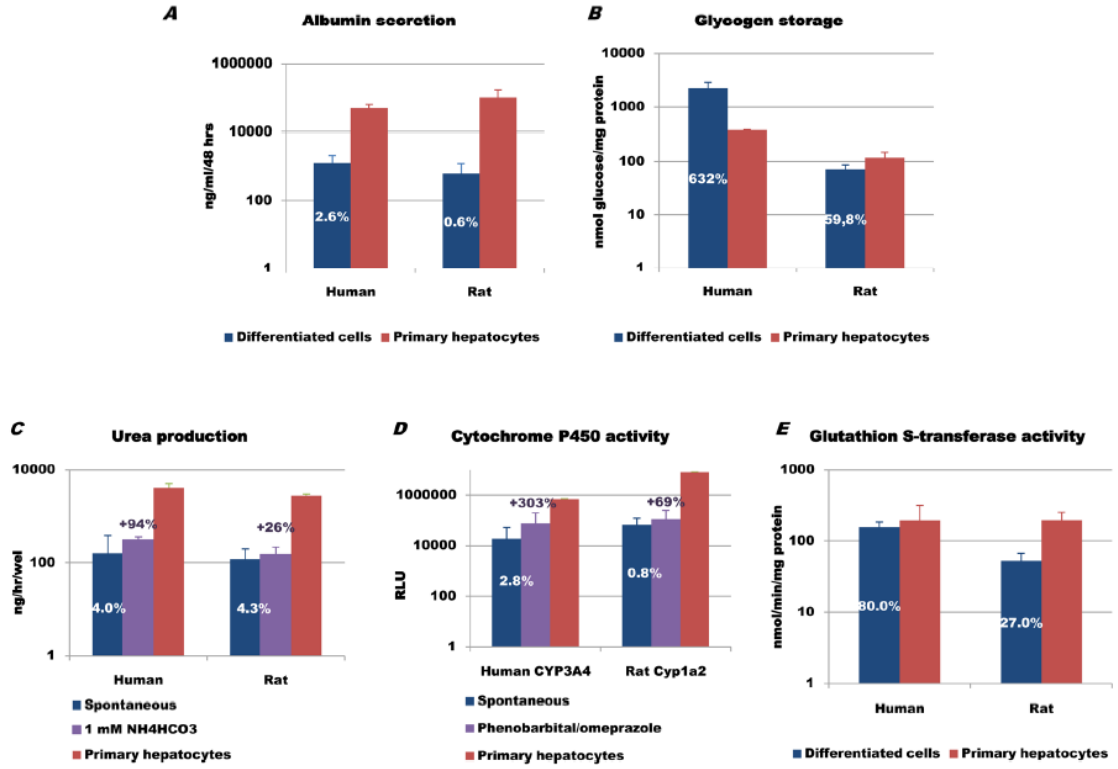
Figure 21. Overview of liver embryogenesis on which liver differentiation protocol is based. Genes specifically expressed at different steps during liver development are shown, in italics, under each step of lineage commitment. Cytokines used as well as other culture conditions are shown. [81]



The functional properties of hepatocytes derived from both human and rodent stem cells are shown in Figure 22. The primary hepatocytes shown in the figure are untreated mature hepatocytes. Human albumin was present in differentiating human ESCs from day fourteen, reaching levels of 2.6 to 5.7% of mature human hepatocytes by day twenty (Figure 22A) [81]. Increasingly more glycogen was stored in the H9 progeny from day six onwards and by day twenty, the glycogen levels were six times greater than short term cultured mature hepatocytes (Figure 22B) [81]. The detoxification functions of the hepatocyte like cells were also evaluated. The cells replied to 1 mM of ammonia by increasing urea production; this increase was detectable from day fourteen and peaked on day twenty (4.0% up to 7.7% after adding ammonium bicarbonate when compared with mature hepatocytes) (Figure 22C) [81]. The expression of cholesterol 7-a-hydroxylase (CYP7A1), a rate limiting enzyme in the production of bile acid from cholesterol, increased in human ESCs progeny by twenty seven times by the twentieth day (reaching levels ten times lower than in mature hepatocytes) [81]. Real time quantitative polymerase chain reaction (RT-qPCR), using primers that recognise cytochrome P 450 3A4/5/7 (CYP3A4/5/7)

transcripts, showed that CYP3A4/5/7 expression levels in human ESC progeny were more highly expressed when compared to foetal human hepatocytes on the twentieth day [81]. The human ESC progeny expressed CYP3A7 at levels found in postnatal liver. On day twenty, CYP3A4/5/7 activity was detected in the human ESC progeny, which was inducible by 500 mM of Phenobarbital [81]. The levels peaked at 2.8% up to 11.1% after the addition of Phenobarbital (when compared with mature hepatocytes) (Figure 22D) [81]. The differentiated human ESC progeny also expressed phase 2 enzymes involved in glucuronidation and sulphation; glutathione-S-transferase (GST) and UDP-glucuronidation (UGT1A1) [81]. On day twenty, GST activity of the human ESC progeny reached levels of 80% of mature hepatocytes (Figure 22E) [81]. The total GST activity was measured using 1-chloro-2,4-dinitrobenzene(CDNB) [81]. In summary, the human ESC progeny demonstrated many of the functional properties of mature hepatocytes.

Figure 22. hESC-H9 and rMAPC-1 progeny display functional properties of hepatocytes on d20 (n .3). The following percentages are functional capacity of hESC- and rMAPC progeny compared to mature human and rat hepatocytes, respectively. [A] Albumin secretion (ng/mL/48 h), hESC 2.6%, rMAPC 0.6%. [B] Storage of glycogen (nmol glucose/mg protein), hESC 632%, rMAPC 59.8%. [C] Spontaneous (hESC 4.0%, rMAPC 4.3%) and NH_4HCO_3-stimulated urea production (hESC 7.7% (+94% induction), rMAPC 5.5% (+26% induction)) [D] Baseline (hESC 2.8%, rMAPC 0.8%) and induced cytochrome P450 activity (hESC 11.1% (+303% induction), rMAPC 1.4% (+69% induction)). 500 mM phenobarbital was used for induction of CYP3A4, while 10 mM omeprazole was used to induce Cyp1a2). [E] Glutathion S-transferase activity (nmol/min/mg protein), hESC 80.0%, rMAPC 27.0% [81]



Strangely, the protocol used to differentiate human ESCs into hepatocyte like cells can also be used for rat multipotent adult progenitor cells (rMAPCs) [81]. However the hepatocyte like cells derived from both cell types is mixed; between 10% and 20% of cells are consistent with late foetal hepatocytes that have achieved storage, synthetic and detoxifying functions similar to those of adult hepatocytes [81]. The functional properties of the derived hepatocytes demonstrated albumin secretion, urea secretion and cytochrome P450 activity consistent or better than observed in reportedly more homogeneous hepatocytes [81]. This differentiation protocol is useful for producing hepatocyte like cells from human and rodent stem cells. Although the cells derived from both human and rodent ESCs demonstrate multiple hepatic functions like glycogen storage, albumin secretion, urea production, CYP450 and GST activity, the levels were consistently

five to ten times lower than in mature hepatocytes. This in vitro procedure obviously does not replicate all signals and interactions present in vivo that manage the coordinated differentiation/maturation from pluripotent stem cells to mature hepatocytes. The development of 3D culture systems may be needed to improve cell-cell interactions and hence allow the production of hepatocytes with fully mature functions and characteristics.

5.4 Extracellular Matrix Techniques for Deriving Hepatocytes from ESCs

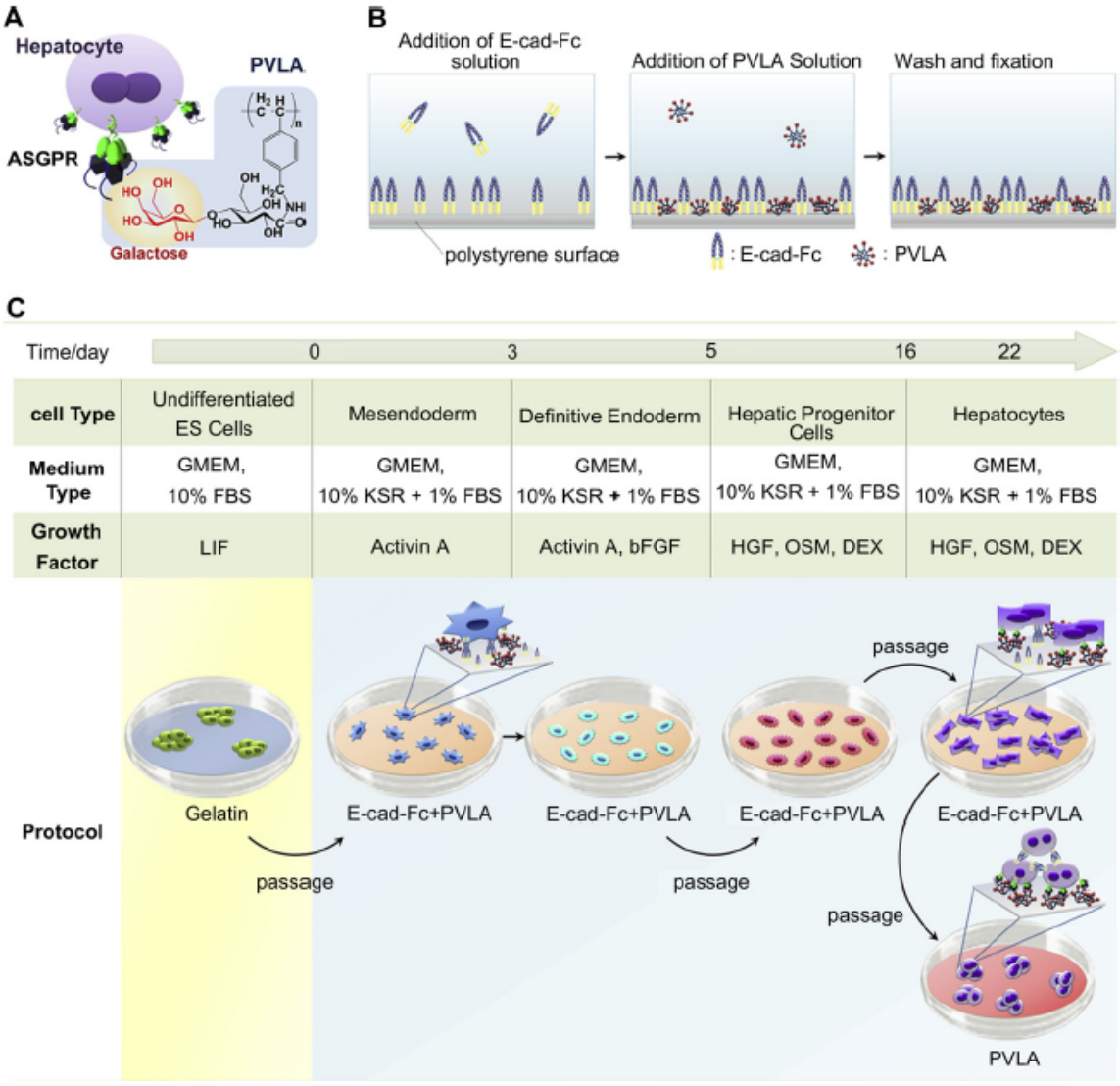
Even if the serum conditions and soluble factors are left unchanged, varying the extracellular matrix is enough to affect human ESC differentiation [3]. The extracellular matrix regulates many cellular processes including differentiation, homeostasis and repair [3]. Each matrix is made up of four main molecular groups: elastin, collagen, proteoglycans and structural glycoproteins [3]. The hepatic extracellular matrix plays an important role in hepatic homeostasis and cirrhosis. Hepatocytes demonstrate a strong polarity in cell shape and cytoskeleton/organelle distribution [3]. Regulating hepatocyte polarity and multicellular organisation is required when developing in vitro systems to carry out liver functions [3]. Optimising the chemical and physical properties of the extracellular matrix is essential for maintaining hepatocyte function in vitro. Pluripotent embryonic cell lines have been used to demonstrate that complex extracellular matrices (like Cartigel and Matrigel) can induce tissue specific differentiation dependent on the type of matrix [82]. This confirms that extracellular matrices can promote ESCs to differentiate into cells and structures that resemble the tissue from which the matrix is derived.

Meng et al differentiated mouse embryonic stem cells into hepatocytes under a monolayer culture condition [65]. The applied extracellular matrix assisted robust and directed differentiation from pluripotent embryonic stem cells into specific lineages of functional hepatocytes [65]. The method used to prepare the PVLA/E-cad-Fc hybrid substratum and induce the embryonic stem cells differentiation to hepatocytes on the co-immobilised matrix is shown in Figure 23. The asialoglycoprotein receptor (ASGPR) is a definitive hepatocyte marker. On the hepatocyte membrane, ASGPR (a type of C-type lectin) carries the glycoproteins in a calcium dependent mode for receptor mediated endocytosis [65]. On the matrices carrying galactose, it is possible to maintain the ASGPR in

a moderately high level on the cellular surface [65]. In hepatocytes, their proliferation signal pathway is triggered by low expression of ASGPR [65]. This represses the differentiation regulations [65]. It was believed that coating the matrix with highly concentrated immobilised poly (N-p-vinylbenzyl-4-O-b-D-galactopyranosyl-D-gluconamide) (PVLA) would sustain the differentiation of primary hepatocytes [65]. The hybrid substratum was developed by initially exploring and establishing the immobilisation of the galactose carrying substratum, E-cadherin-IgG Fc (E-cad-Fc) and PVLA [65]. This was done on the polystyrene dish by using quartz crystal microbalance (QCM) evaluation [65]. PVLA and E-cad-Fc were confirmed to be firmly co-adsorbed onto the surface of the dish [65]. The PVLA/E-cad-Fc artificial substratum was proven efficient in culturing primary hepatocytes and sustaining liver functions [65]. The undifferentiated embryonic stem cells also maintained a high proliferative ability [65]. The derived hepatocytes on the artificial matrix expressed an increased level of liver specific functions and genes as well as early expression of the asialoglycoprotein receptor (ASGPR) [65]. The last step of this technique was isolating a large percentage of cells (around 60%) with ASGPR expression after re-seeding onto a surface coated with PVLA [65]. The eradication of the inadequately differentiated cells (Sox17+ and Gata6+) and the ones toward other cell lineages (Pdx1+ and brachyury+) was observed [65]. This particular system utilises a glycopolymer as an extracellular substratum for isolating and enriching the hepatocytes derived from embryonic stem cells. This gives the hepatocytes adequate homogeneity and functionality [65]. This simple protocol could allow the production and scaling up of embryonic stem cell derived hepatocytes in quantities that could be used in medical applications.

Figure 23. Schematic diagram of the technique used to prepare the PVLA/E-cad-Fc hybrid substratum and induce the embryonic stem cells differentiation to hepatocytes on the co-immobilised matrix. [A] shows the PVLA polymer's molecular formula where the galactose residues bind to the ASGPRs on the hepatocyte membranes. [B] illustrates the preparation method for the co-coating layer of E-cad-Fc and PVLA on the polystyrene surface. The [C] diagram of the protocol for the differentiation of mouse embryonic stem cells to hepatocytes includes the states of cellular undifferentiation (yellow area) and differentiation (blue area). Cellular differentiation is split into four different stages: mesendoderm, definitive endoderm, hepatic progenitor cells and finally hepatocytes. [65]

Abbreviation: bFGF: basic fibroblast growth factor; HGF: hepatocyte growth factor; LIF: leukemia inhibitory factor; DEX: dexamethasone; OSM: oncostatin M [65]



5.5 Epigenetic Control of Gene Transcription Techniques for Deriving Hepatocytes from ESCs

Other studies have examined the epigenetic control of gene transcription; including DNA (de)methylation, histone (de)acetylation and transcription factors [61]. Pluripotent embryonic stem cells and more lineage restricted adult stem cells (or progenitor cells) vary in their global gene expression profile [61]. Stemness genes are active in pluripotent ESCs and are gradually silenced and lineage specific genes are activated as development progresses [61]. This inconsistency in gene expression status might be attributed to modifications in the nuclear and chromatin structure, resulting in limited access of transcription factors in the direction of specific DNA binding locations [61]. A dynamic interaction between epigenetic regulators and a range of lineage specific transcription factors is a feature of each step [61]. The base of a stem cell's identity is formed by the epigenetic code [61]. This code determines how responsive a cell is to extrinsic signals at consecutive developmental stages [61]. Song et al believe that extracellular growth factors may affect the chromatin status and assist or hinder the differentiation capability of stem cells [83]. The four transcription factors (Oct4, Sox2, c-Myc and Klf4) perform as regulators of the transcriptional circuitry to sustain pluripotency of embryonic stem cells. A summary of applied epigenetics based techniques for in vitro hepatic differentiation of embryonic stem cells is given in Figure 24 [61]. Spontaneous embryonic stem cell differentiation can occur due to a lack of organisation and inherent diversity [61]. In order to derive specific cell populations; biologically derived signals (like purified growth factors), chromatin remodelling agents and other lineage selective agents have been introduced [61]. Exposing embryonic stem cells to 5 mM of sodium butyrate leads to 10% to 15% differentiating into pure hepatic cells [61]. Between 10% and 70% enrichment can be achieved by treating the stem cells with alternating concentrations of sodium butyrate (between 0.5 and 1 mM) in the presence of activin A [61]. In summary, combining epigenetic modification with steps of exposure to cytokines considerably enhances the homogeneity of the end cell population and attainment of hepatic functionality [61].

Figure 24. Overview of epigenetics based techniques for in vitro differentiation of ESCs into hepatic cells [61]

Origin	Hepatic differentiation conditions					Hepatic features	
	Cell density	Cell–matrix/ cell–cell interaction	Serum	Growth factors-cytokines nonepigenetic additives	Differentiation-inducing agents	RNA + protein level	Functionality level
EMBRYONIC STEM CELLS							
				Hepatic progenitor cells			
	NS	Gelatin	15% FBS	(3)*D11–17/23:* 10 ng/ml HGF until confluent	(1)*D0–4*: 0.8% DMSO (2)*D4–10*: 2.5 mM SB	AFP, α1AT, CK18/19 GGT, HNF3 β, DPPIV	
mES(D3)				**Hepatocytes**			
	NS	Collagen type I	10% FBS	4)*D17/23–23/29:* insulin, dex nicotinamide 20 ng/ml EGF 10 ng/ml HGF (5)from D23/29: 10 ng/ml OSM, dex		ALB, G6P↑, TAT↑	Glycogen storage ALB secretion
hES	NS	PAU-coating, nonwoven PTFE	NS	*D0–3*:100 ng/ml bFGF *D3–11*:100 ng/ml HGF *D11–14*:dex	*D3–11: 1%* DMSO	ALB	Lidocaine metabolism ALB and urea production
hEBs		Matrigel	20%FBS		5 mM SB	−: AFP +: ALB, α1AT, CK8/18	Glycogen storage Inducible CYP450 activity
mES (D3)	1×10^4 cells/cm^2	*D0–10*:Gelatin collagen type I polystyrene	*D0–10*: 20% FBS		*D0–4*: 1% DMSO *D4–10*: 2.5 mM SB	+: ALB, K18, DPPIV ADH, CYP3A13, CYP27A1	Glycolysis Glycogen storage Urea production CYP activity
	1×10^4 cells/cm^2 D11: subculture at 15×10^3 cells/cm^2	*D0–10:* gelatin from D11: 5% polyacrylamide	*D0–10:*20% FBS from D11:/		*D0–4:* 1% DMSO *D4-10:* 2.5 mM SB From D11:/		Glycogen storage ↑urea production ↑ALB secretion
mES (D3)	Phase I Pre-differentiation = no confluence	Gelatin	15%FBS	(3) from D10 until confluence: 10 ng/ml HGF	(1)*D0–4*: 0.8% DMSO (2)*D4–10:* 2.5 mM SB	+: CK19, ALB, α1AT HNF3*β* -: SSEA1, AFP	
	Phase II Differentiation upon confluence and subculture	Collagen type I	10%FBS	(4) Upon subculture: *D0–6*: insulin, dex nicotinamide, 20 ng/ml EGF 10 ng/ml HGF (5) From *D6–12/18*: (5) From *D6–12/18*: 10 ng/ml OSM, dex		+: AFP, ALB, CK18, α1AT HNF3*β*, HNF4, TAT -: CK19	Glycogen storage ALB secretion
hES	Differentiation onset pon 50–70% confluence D3/5:1/2 split	Matrigel	*D0–3/5:*/ *D3/5–D10/12*:SR from *D10/12*: 8.3% FBS	*D0–3/5:* 100 ng/ml activin A From D10/12:insulin, hydrocortisone, 10 ng/ml HGF, 20 ng/ml OSM	*D0-D1/2*:1 mM SB *D1/2–3/5*: 0.5 mM SB *D3/5–D10/12*: 1% DMSO	*D0–3/5:* CXCR4, HNF3*β*, Sox17 D3/5–D10/12: HNF4α↑, HNF1↑, TTR↑ AFP From *D10/12:*ALB, ApoF, CAR, TO, TAT↑, CYP3A4/7, CYP2C9/19	Glycogen storage ALB/fibrinogen/ fibronectin/A2M secretion inducible CYP activity
mES Monkey ES	mES:10^9 cells/ cm^3 lumen V monkey ES: 4×10^3 cells/cm^3 lumen V	Organoid culture in hollow fibers	20% FBS		*D9*: 1 mM SB	+: CPS↑	ALB secretion ammonia removal

Abbreviation: a1AT, alpha1-antitrypsin; ADH, alcohol dehydrogenase; ADSC, adipose tissue-derived stem cells; AFP, alpha-fetoprotein; ALB, albumin; A2M, a2macroglobulin; ApoF, apolipoprotein factor; 5-AzaC, 5-azacitidine; bFGF, basic fibroblast growth factor; BM, bone marrow; CAR, constitutive androstane receptor; C/EBP, CCAAT enhancer binding protein; CK, cytokeratin; CPS CPS-1, carbamyl phosphate synthetase; Cx, connexin; CYP, cytochrome P450-dependent monooxygenases; dex, dexamethasone; DMSO, dimethylsulfoxide; DPPIV, dipeptidylpeptidase IV; EGF, epidermal growth factor; ES, embryonic stem cells; FBS, fetal bovine serum; FCS, fetal calf serum; FGF, fibroblast growth factor; FN, fibronectin; GGT, c-glutamyltransferase; G6P, glucose-6-phosphatase; h, human; HepPar1, hepatocyte paraffin 1; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; ITS, insulin-transferrin-selenious acid; LDL, low density lipoprotein; m, mouse/murine; MRP, multidrug resistance protein; MSC, mesenchymal stem cells; NS, not specified; OSM, oncostatin M; P, passage; PAU, poly-amino-urethane; PEPCK, phosphoenol-pyruvate carboxykinase; PTFE, polytetrafluoroethylene; SB, sodium byturate; SD, Sprague–Dawley; Sox17, Sry-related HMG box transcription factor; SR, serum replacement; SSEA, stage-specific embryonic antigen; TAT, tyrosine amino-transferase; TERT, telomerase reverse transcriptase; TO, tryptophan-2,3-dioxygenase; TSA, trichostatin A; TTR, transthyretin; UCB, umbilical cord blood; 1.,2.,3; indicate order of serial steps;↓, downregulation;↑, upregulation; -, negative; +, positive. [61]

5.6 Conclusion

Human embryonic stem cells can spontaneously form embryoid bodies. These bodies then spontaneously differentiate to various cell lineages (specific to the tissue) containing a total of 10% to 30% albumin producing hepatocytes and cells like hepatocytes [66]. When the embryoid bodies are enriched with definitive endoderm, the final ratio of hepatocyte population is increased up to 50% to 65% [66]. Existing techniques for the directed differentiation of human embryonic stem cells to hepatocytes that reproduce liver embryogenesis by stepwise stimulation of culturing ESCs with liver specific growth factors achieve a differentiation rate of between 60% and 80% [66]. Several groups are trying to develop ESC differentiation protocols that try to replicate normal embryonic development. The initial step of hepatic development uses activin A to induce definitive endoderm [64]. However the derived cells often fail to function sufficiently for regenerative therapy, they remain hepatocyte like cells [64]. Directed differentiation of human embryonic stem cells to hepatocytes needs further improvement to allow the production of homogeneous cultures of hepatocytes; this will avoid expensive methods of hepatocyte (and hepatocyte like cell) isolation and separation [66]. Ideally the specialised tissue microenvironment for stem cells would be three dimensional (3D) [3]. The main problem associated with two

dimensional (2D) cellular assays, is that they cannot replicate the in vivo response of cells in the 3D environment found in tissues [3]. Perhaps 3D modelling could be used to gather information on cellular interactions and physiology [3]. This information could then be converted to 2D in vitro models [3]. Care must be taken when identifying hepatic cells because most of the genes expressed in the liver like phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) are also present in other tissues such as yolk sac tissue [62]. This is why discrete markers for hepatocytes and yolk sac tissue are required to distinguish between these two cell populations [62]. CYP7A1 is used as a marker for hepatocytes derived from ESCs because it is expressed in the liver but not expressed in the yolk sac tissue [62]. A lot of progress has been made in the differentiation of human embryonic stem cells to hepatocyte like cells. However ethical issues limit the availability of human embryonic stem cells. Concerns have been raised about human embryonic cells in terms of safety, efficacy and ethics. Before human ESCs can be safely used in clinical trials, more studies are required. The risks associated with ethical issues and immune rejection have resulted in a lot of focus being placed on other types of pluripotent stem cells like induced pluripotent stem (iPS) cells [62] [66].

6. iPSC Derived Hepatocytes

6.1 Introduction

As mentioned previously, induced pluripotent stem cells (iPSCs) are produced by introducing one or more genes to adult stem cells and characterised by multi-lineage differentiation capability. iPSCs have been used to produce a variety of differentiated cells including neurons, cardiomyocytes and hepatocytes [84]. Directed cell differentiation from ESCs could be used to obtain hepatocytes in vitro but this source is severely limited by ethical and legal issues. These issues have led to a lot of focus being put on human iPSC derived hepatocytes. iPSCs are similar to ESCs in phenotype and functionality, particularly their ability to differentiate into various specific cell lineages. In the past few years, a selection of strategies has been developed for deriving hepatocytes from iPSCs.

6.2 Techniques for Deriving Hepatocytes from iPSCs

In 2009, Song et al were the first to describe the successful differentiation of human iPSCs into hepatocyte like cells [85]. Song et al used a stepwise procedure to induce human iPSCs to differentiate into hepatic cells [85]. The expression of hepatocyte cell markers and liver specific functions of the human iPSC derived cells were monitored [85]. These results were then compared with those of differentiated from human ESCs. After seven days, around 60% of the differentiated human IPSCs expressed hepatic markers α-fetoprotein and albumin [85]. On the twenty first day, the differentiated cells exhibited hepatocyte cell functions including albumin secretion, urea production, glycogen synthesis and inducible cytochrome P450 activity [85]. The expression of the hepatic markers and liver related functions of the iPSC derived hepatic cells were similar to that of the human ESC derived hepatic cells [85]. Other reports of successful iPSC hepatic differentiation followed soon after, each with their own unique 'recipe' (Rashid et al [86], Gai et al [87], Sullivan et al [88] and Si Tayeb et al [89]).

The efficient generation of hepatic endodermal lineage from human iPSCs was first demonstrated by Sullivan et al in 2010 [88]. 1×10^5 fibroblasts from a normal Caucasian male were plated in one well of a well plate [88]. They were then infected with four

individual retroviruses with a multiplicity of infection (MOI) of 10 [88]. MOI is the number of infectious virus particles that are introduced per target cell during infection. If ten infectious virus particles are added for every target cell, MOI is 10. MOI of 10 is used when the study requires every target cell to be infected. Each of the viruses contained one reprogramming factor (Oct4, Sox2, Klf4 and c-MYC) [88]. After three days of infection, the cells were split into 10 cm plates [88]. The plates were pre-seeded with irradiated mouse embryonic fibroblasts and cultured under human ESC culture medium conditions until colonies appeared [88]. These colonies were then picked, re-plated onto irradiated mouse embryonic fibroblasts and expanded for characterisation [88]. KnockOut D-MEM (KO-DMEM) is a basal medium optimised for growth of undifferentiated IPSCs and ESCs [90]. The osmolarity of KO-DMEM is optimised to estimate that of mouse embryonic tissue [90]. The iPSC colonies were maintained in human ESC medium with 80% KO-DMEM (20% KO Serum Replacement, 100 μM of β-mercaptoethanol, 1 mM of L-glutamine, 100 mM of non-essential amino acids, 50 U/ml of penicillin, 10ng/ml of bFGF and 50 mg/ml of streptomycin on an irradiated mouse embryonic feeder layer) [88]. The iPSCs were cultured on Matrigel before hepatic endoderm differentiation [88]. They were differentiated to cells (like hepatocytes) using activin A and Wnt3a on Matrigel [88]. The protocol used was similar to the one described for ESCs by Hay et al in 2008 [71]. One main alteration was required to produce human hepatic endoderm from human iPSCs; after the iPSCs were passaged onto Matrigel and cultured in mouse embryonic fibroblast conditioned medium (MEF-CM) until a confluence of 50% to 70% was achieved, MEF-CM was then replaced with culture medium, RPMI-B27 [71]. The iPSCs were treated with activin A and Wnt3a for three days and needed a further two day incubation in only activin A (100 ng/ml) before hepatic endoderm was specified using the following conditions: cells were cultured in SR-DMSO (KO-DMEM containing 20% serum replacement, 1% DMSO, 1% non-essential amino acids, 0.1 mM of β-mercaptoethanol and 1 mM of glutamine) [88]. The final maturation stage involved culturing the cells in L-15 medium supplemented with 8.3% tryptose phosphate broth, 8.3% foetal calf serum, 10 μM of Hydrocortisone 21-hemisuccinate, 1 μM of insulin, 2 mM of glutamine, 10 ng/ml of HGF and 20 ng/ml of OSM [88]. This study proved that iPSC lines can be differentiated to hepatic endoderm at efficiencies of between 70% and 90% [88]. The iPSC derived hepatic endoderm displayed hepatic morphology and also expressed the hepatic markers; E-Cadherin and albumin [88]. They also expressed α-foetal protein (AFP), HNF4α and Cyp7A1 (metabolic marker) [88]. This demonstrated a definitive endodermal

lineage differentiation. The iPSC derived hepatocytes generated and secreted plasma proteins, fibronectin, fibrinogen, AFP and transthyretin (TTR) [88]. The iPSC derived hepatic endoderm supported both CYP3A4 and CYP1A2 metabolism (essential for drug and toxicology testing) [88]. This study illustrated that iPSCs could become fully functional hepatic cells because the iPSC derived hepatocytes expressed distinct hepatocyte markers and displayed definitive function.

Also in 2010, Rashid et al took dermal fibroblasts from patients suffering from various inherited metabolic liver diseases and used them to fabricate a collection of human iPSC lines specific to each patient [86]. Each cell line was then differentiated into hepatocytes using a three step differentiation protocol based on chemically defined conditions [86]. Initially 8 mm skin punch biopsies were taken from volunteering patients [86]. Fibroblasts were then derived from the donated tissue under good manufacturing practice conditions [86]. Further fibroblast samples were given by INSERM and Coriell Biorepository [86]. There was a total of five different disease samples (obtained from seven different patients) [86]. Vectors derived from Moloney Murine Leukemia, each containing one of the four human gene (OCT4, SOX2, c-Myc, and KLF4) coding sequences were used to induce pluripotency [86]. The fibroblasts were infected with the corresponding viral particles at multiplicity of infection of ten [86]. After derivation, the human iPSCs were cultured in standard human ESC culture conditions; KnockOut Serum Replacement and 4 ng/ml of FGF2 on plates precoated with irradiated mouse feeder cells [86]. The iPSC derived cells exhibited properties of mature hepatocytes including albumin secretion and metabolism of cytochrome P450 [86]. However the cells generated also displayed key pathological features associated with the diseases that affected the patients (from which the iPSCs were derived) [86]. These patient derived hepatocytes could be used to model inherited metabolic liver diseases and disorders but would obviously not be suitable for cellular therapies.

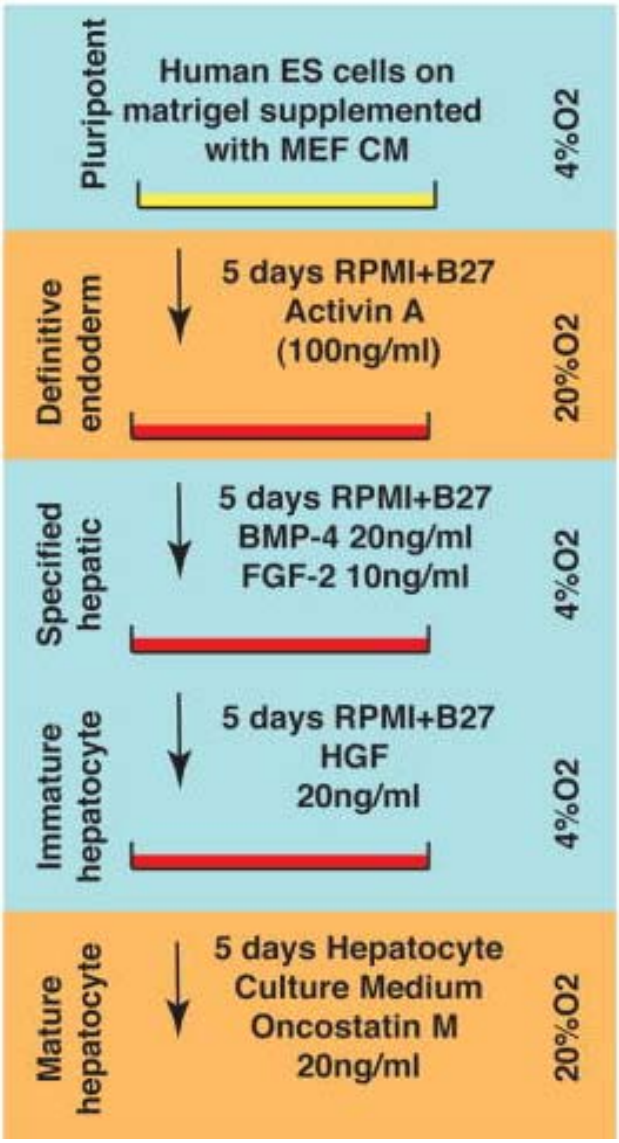
Also in 2010, Gai et al developed a stepwise procedure to induce murine hepatic differentiation from iPSCs [87]. Induced pluripotent stem cells were generated from adult stem cells by retrodifferentiation (using defined transcription factors) [87]. The iPSCs were reprogrammed from mouse tail tip fibroblasts [87]. After treatment with 0.25% trypsin-EDTA, the mouse iPSC colonies became a single cell suspension [87]. Embryoid bodies were fashioned by the hanging drop method in Dulbecco's modified eagle medium (DMEM)

supplemented with 0.1 mM of β-mercaptoethanol, 2 mM of glutamine, 20% foetal bovine serum (FBS) and 1% minimum essential medium (MEM) non-essential amino acid solution for two days [87]. The embryoid bodies were relocated to petri dishes for an additional four days in suspension [87]. They were then plated to tissue culture dishes coated with Matrigel in DMEM with 50 ng/ml of activin A, 50 ng/ml of Wnt3 and 10% FBS [87]. The following day (when most of the embryoid bodies were attached), the cultures were moved to DMEM supplemented with 50 ng/ml of activin A, 50 ng/ml of Wnt3 and 1% FBS [87]. The medium was changed every day for the next three days [87]. To further instigate hepatic differentiation, DMEM supplemented with 10ng/ml of bFGF, 10% FBS and 1% dimethylsulfoxide (DMSO) was used in the cultures for four days and the following five days used 10 ng/ml of HGF and 1% DMSO in hepatocyte culture medium (HCM) [87]. To improve hepatic maturation, the cultures were fed with HCM with 10 ng/ml of HGF, 10 ng/ml of OSM and 0.1 mM of DEX for the final seven days [87]. On day twenty five of differentiation, the hepatocytes derived from iPSCS resembled mouse primary hepatocytes in terms of phenotype (with a distinct polygonal shape) [87]. The derived cells were analysed using reverse transcription polymerase chain reaction analysis and immunostaining [87]. These analyses revealed that the cells had expressed specific hepatic markers including albumin, α-fetoprotein and α-1-anti-trypsin [87]. The iPSC derived hepatocytes also exhibited mature liver cell functions in vitro and successfully engrafted into the recipient livers in vivo [87].

Si Tayeb et al demonstrated in 2011 that hepatic cells could be generated by co-culturing mouse iPSCs with human ESCs in a standard embryonic stem cell maintenance media [89]. The aim of this work was to create a protocol that did not have any steps that introduced poorly defined components into the culture conditions because this could be problematic in a cellular therapy [89]. This method did not use serum, embryoid bodies, fibroblast feeder cells or undefined culture medium components [89]. The protocol used is based upon understanding the mechanisms that make up mouse embryogenesis. The mouse iPSCs were fabricated from fibroblasts [89]. The procedure used is shown in Figure 25 [89]. The undifferentiated stem cells were kept in monolayer culture on Matrigel in ESC culture media supplemented with mitotically inactivate primary mouse embryonic fibroblasts in 4% oxygen (O2) and 5% carbon dioxide (CO2) [89]. More than 95% of the cells expressed pluripotency markers, under these conditions [89]. To start hepatic differentiation, monolayers of ESCs were cultured in Roswell Park Memorial Institute (RPMI) media

containing B27 supplements and 100 ng/ml of activin A [89]. More than 90% of the cells had lost expression of the pluripotency markers OCT3, OCT4 and SSEA4, after five days of culture in 5% carbon dioxide and ambient oxygen [89]. More than 80% of the cells expressed GATA4, FOXA2 and SOX17 but did not express HNF4α (highly expressed in extra embryonic endodermal cells) [89]. This lack of expression eliminated any concerns that the endoderm generated by activin A was yolk sac (visceral) endoderm [89]. The culture dishes that contained induced definitive endoderm were then moved to 5% CO2 and 4% O2 in RPMI-B27 media complemented with 10 ng/ml of FGF2 and 20 ng/ml of BMP4 for five days [89]. FGF2 and BMP4 both play crucial roles throughout hepatic specification in mouse embryos. After the addition of FGF2 and BMP4, the expression of both GATA4 and SOX17 was reduced, while the expression of FOXA2 was maintained and HNF4α expression was initiated [89]. Following the introduction of FGF2 and BMP4, more than 80% of cells expressed HNF4α [89]. This pattern of GATA4, SOX17, FOXA2 and HNF4α expression is very similar to that found during mouse liver development; GATA4 expression is reduced specifically in cells that are destined to follow a hepatic fate but maintains expression in gut endoderm, the expression of HNF4α is limited to the developing hepatic cells formed during the hepatic specification stages of development [89]. The specified hepatic cells were cultured in RPMI-B27 supplemented with 20 ng/ml of HGF, under 5% CO2 and 4% O2 [89]. The inclusion of HGF in the culture conditions produced high levels of α-fetoprotein expression, which indicated that the specified cells had committed to a hepatoblast fate [89]. Co-staining with FOXA2 revealed that more than 98% of FOXA2 expressing cells also expressed α-fetoprotein meaning that differentiating endoderm into hepatic lineage was a very efficient process [89]. The last stage of differentiation involved transferring cultures to 5% CO2 and ambient O2 as well as replacing the media with HCM supplemented with 20 ng/ml of OSM for an additional five days [89].

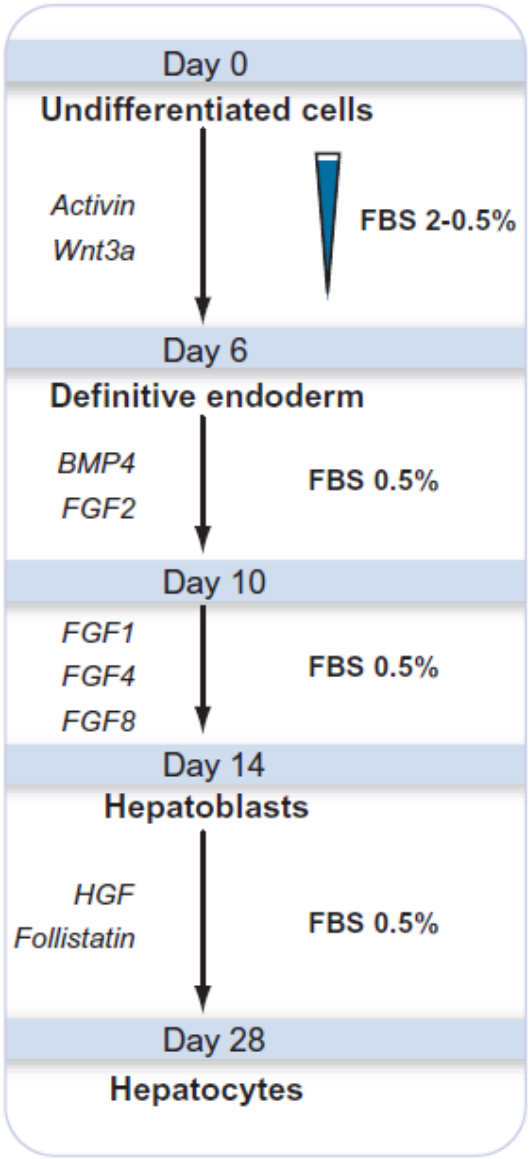
Figure 25. Flow diagram summarising Si Tayeb et al's protocol for controlling hepatocyte differentiation [89]



The cells expressed high levels of albumin under these conditions [89]. Based on flow cytometry analyses, around 80% of cells were albumin positive [89]. After the differentiation protocol, the generated cells exhibited several hepatic functions including glycogen synthesis, accumulation of LDL, uptake of indocyanine green and urea metabolism [89]. The phenotype of the differentiated cells also shared a lot of characteristics with primary hepatocytes including prominent nucleoli, large cytoplasmic to nuclear ratio as well as numerous vacuoles and vesicles [89]. This procedure allows the efficient production of highly differentiated human hepatocyte like cells from iPSCs. The hepatocyte like cells exhibit key liver functions and can engraft into hepatic parenchyma in vivo [89]. This report concluded that mouse iPSCs retain full potential for foetal liver development [89].

Sancho Bru et al reported in 2011 that a stepwise protocol (modelled on embryonic liver development) induced hepatic differentiation of mouse iPSCs and produced a population of cells with mature hepatocyte phenotype [91]. Sequential stimulation with cytokines was used to differentiate the iPSCs. The iPSCs were induced to primitive streak then mesendoderm followed by definitive endoderm [91]. The definitive endoderm cells were then differentiated into cells with hepatoblast characteristics then finally into those with hepatocyte features [91]. A diagram of the differentiation protocol used by Sancho Bru et al can be seen in Figure 26 [91]. Before initiating hepatic differentiation, the undifferentiated iPSCs were plated on gelatin with no mouse embryonic fibroblasts in ESC medium [91]. To initiate hepatocyte differentiation, mouse iPSCs were plated in well plates precoated with 2% Matrigel and diluted in phosphate buffered saline for one to two hours at 37 ^{o}C, shortly before plating the cells [91]. On day 0, the cells were seeded at 2500 cells per cm^{2} in a differentiation medium [91]. The differentiation medium consisted of 60% DMEM, 40% MCDB-201-water, low glucose, 0.25% insulin transferring selenium, 0.25% linoleic acid bovine serum albumin, 100 μg/ml of streptomycin, 100 IU/ml of Penicillin, 10-4 M of L-ascorbic acid, 10-6 M of DEX and 100 μM of β-mercaptoethanol [91]. During differentiation, the well plates were kept at 21% O2 and 5.8% CO2 in a humidified incubator [91]. The medium was changed 70% every second day and entirely when the medium components were changed (days 6, 10 and 14) [91]. 2% FBS was added on days 0 to 2 then 0.5% FBS from day 3 to 28 [91]. The cytokines were introduced as follows: 100 ng/ml of activin A and 50 ng/ml of Wnt3a from day 0 to day 6, 50 ng/ml of BMP4 and 10 ng/ml of FGF2 from day 7 to day 10, 50 ng/ml of aFGF, 10 ng/ml of FGF4 and 25 ng/ml of FGF8b from day 11 to 14 and finally 20 ng/ml of HGF and 100 ng/ml of follistatin from day 15 to 28 [91].

Figure 26. Diagram displaying Sancho Bru et al's differentiation protocol [91]



The hepatocyte like cells displayed functional properties of hepatocytes including albumin secretion, urea production, glycogen storage and inducible cytochrome activity [91]. The mesodermal cells also displayed some functions associated with liver sinusoidal endothelium and stellate cells [91]. This report concluded that a procedure (modelled on embryonic liver development) can be used to induce the hepatic differentiation of mouse iPSCs, resulting in a population of cells with mature hepatic phenotype [91]. However, these cells failed to expand or contribute significantly to liver regeneration when transplanted into an animal model [91]. This suggests that the iPSC derived cells can engraft in the liver but have limited ability when it comes to proliferation and regeneration.

6.3 Conclusion

Most of the protocols for differentiating iPSCs into hepatocytes are very similar to ESC protocols; the iPSCs are initially induced to differentiate into definitive endoderm or embryoid bodies. The most successful strategies sequentially introduce endodermal, small molecules and hepatic inductive cytokines to replicate stepwise liver embryogenesis. These methods usually result in iPSC derived hepatocytes demonstrating many key characteristics of primary human hepatocytes. Although no techniques have reported the production of cells that completely demonstrate all features and functions of primary human hepatocytes, human iPSC derived hepatocytes show a similar gene expression profile (compared to primary hepatocytes), secrete functional proteins and metabolise chemical substances in vitro. However compared to primary hepatocytes, the iPSC derived hepatocytes secrete less albumin and display lower levels of P450 enzyme activities. The human iPSC derived hepatocytes consistently express the foetal stage hepatocyte marker, α-fetoprotein; this suggests that the hepatocytes derived are at an immature or foetal stage of development. The iPSC hepatocytes have demonstrated their ability to engraft and mature in animal models [89]. This is probably the best way of proving their identity and functionality. Reprogramming a patient's own cells (autologous) could make iPSC hepatocytes a better source for cell therapy because there would be much less chance of immune rejection. iPSCs originate from adult stem cells and therefore avoid a lot of the ethical issues associated with human embryonic cells. The generation of iPSC derived hepatocytes from a patient would also bear the inherited pathological characteristics. This could potentially allow disease modelling and drug development to be more specific to each patient. Although developing drugs for the specific needs of individual patients would never be financially worthwhile for pharmaceutical companies. Inheriting a patient's pathological characteristics could well be a disadvantage in cellular therapies if the cause of the patient's liver failure is inherited not induced (like Biliary Atresia or Autoimmune Hepatitis) because this may mean that the iPSC derived hepatocytes would naturally mature into faulty cells.

7. MSC Derived Hepatocytes

7.1 Introduction

There are a large number of potential sources of MSCs in the human body including: adipose tissue, umbilical cord blood, olfactory mucosa, bone marrow, articular cartilage, muscle and other tissues. In 2008, Banas et al concluded that human MSCs could promote liver repair when transplanted into mice with liver injury [92]. This suggests that MSCs can differentiate into functional hepatocytes and therefore could be useful sources for replacing damaged liver tissue.

7.2 Techniques for Deriving Hepatocytes from MSCs

In 2004, Lee et al examined whether mesenchymal stem cells (MSCs) could differentiate into functional cells similar to hepatocytes in vitro [93]. MSCs were isolated from umbilical cord blood and human bone marrow [93]. A two step protocol involving HGF and OSM was used to induce hepatic differentiation [93]. The cells displayed cuboidal morphology (characteristic of hepatocytes) and expressed liver specific marker genes in a time dependent manner after four weeks of induction [93]. The differentiated cells also demonstrated in vitro hepatocyte functions including glycogen storage, urea secretion, albumin production, uptake of LDL, and inducible cytochrome P450 activity [93]. In conclusion MSCs from both sources could differentiate into cells similar to functional hepatocytes [93].

In 2010, Waclawczyk et al used a three step differentiation procedure that mimicked the embryonic developmental processes of hepatic endoderm [94]. This research used MSCs taken from human umbilical cord blood [94]. The SOX17 and FOXA2 expression of the cells was enhanced by activin A and FGF4 [94]. Further hepatic differentiation to hepatic endoderm (characterised by the expression of AFP and HNFα) was completed sequentially by retinoic acid, HGF, FGF4 , EGF, and OSM [94]. Progressive differentiation was indicated by the expression of HNFα, G6PC, ARG1 and FBP1 [94]. The cells demonstrated hepatocyte functions including urea formation, albumin secretion and CYP3A4 enzyme activity [94]. Demonstrating these functions confirmed that these cells were hepatic like [94].

Waclawczyk et al concluded that MSCs taken from human umbilical cord blood represented a suitable cell source with the capability for hepatic differentiation [94].

In 2012 Yu et al analysed the hepatic differentiation potential of mesenchymal stem cells (derived from fresh human umbilical cord blood) after transplantation into severe combined immune deficiency mice with liver injury [95]. The MSCs were positive for the human MSC specific markers CD29, CD73, CD90, CD105 and CD271 but negative for CD34 (hematopoietic lineage marker) and CD45 (leukocyte common antigen) [95]. The lack of CD34 and CD45 expression suggested that these cells were not of hematopoietic origin [95]. Liver injury was induced by D-galactosamine/lipopolysaccharide [95]. As well as the hepatic differentiation potential, this report also examined if the cells could repair hepatic damage [95]. Mononuclear cells were isolated from human umbilical cord blood and then transferred into the injured mice [95]. Specimens were collected every seven days (on days seven, fourteen, twenty one and twenty eight) after transplantation [95]. The cells expressed human hepatocyte specific markers like human albumin, human α-fetoprotein, human cytokeratin 18 and human cytokeratin 19 seven days after MSC transplantation [95]. The expression of human α-fetoprotein was no longer detected twenty one days after transplantation [95]. These markers suggested the formation of hepatocyte like cells and were analysed by reverse transcriptase and immunohistochemical staining [95]. After human MSC transplantation, histological investigations found that the hepatic damage in mice was lessened and the liver structure was better preserved [95]. Seven days after MSC transplantation, less hepatic damage was detected and cell regeneration was apparent [95]. After twenty eight days, hepatic endoderm staining revealed almost normal liver lobular architecture and inflammatory cell infiltration, hepatocyte denaturation and necrosis had just about disappeared [95]. Yu et al concluded that MSCs from fresh human umbilical cord blood demonstrate the potential to differentiate into cells similar to hepatocytes and partially repair the induced liver damage [95].

7.3 Conclusion

MSCs can be derived from different body parts and have the ability to differentiate into cells of different tissues. This makes them a potentially excellent source for cellular therapy. Using autologous adult stem cell derived hepatocytes for transplantation would

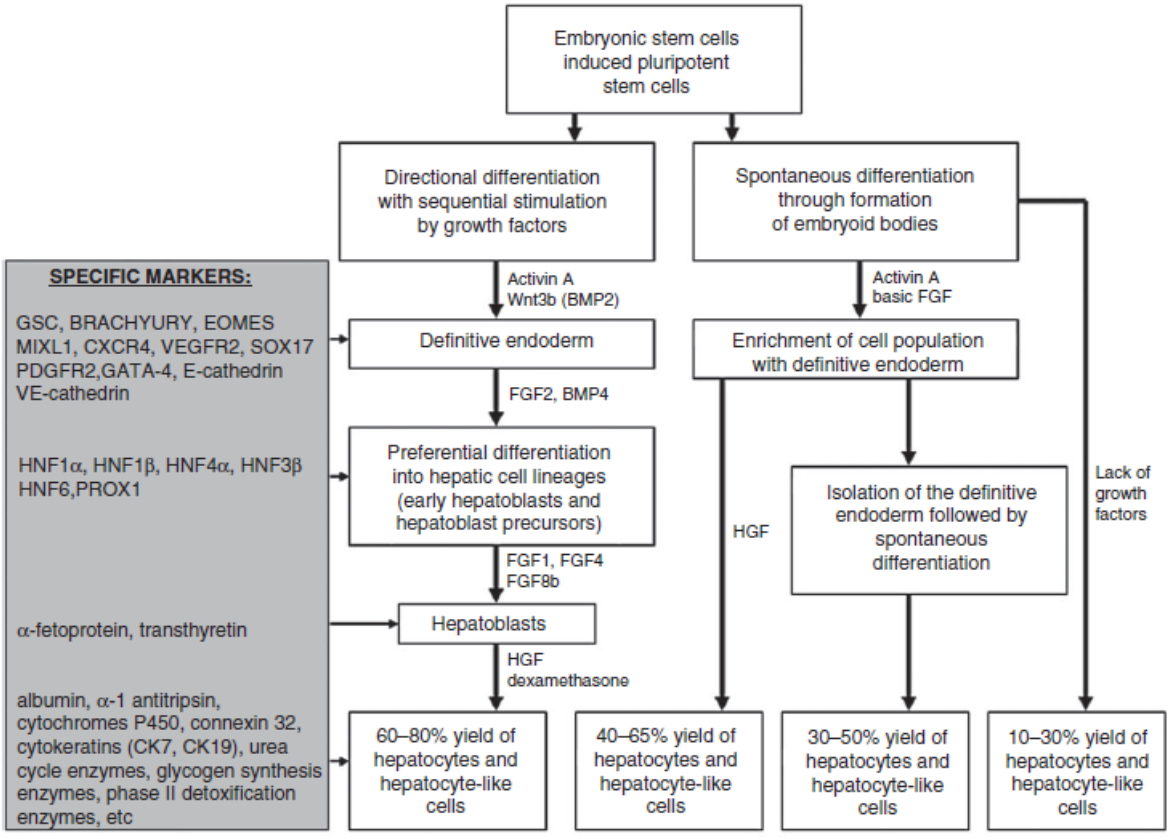
avoid many ethical issues. They would also be better than ESCs in terms of immune system rejection. One disadvantage of MSCs is the lack of significant improvement made in differentiation protocols between 2004 and 2012. It is still unknown if MSC derived hepatocyte like cells can carry out all of the functions required by the liver.

8. iPSC and ESC Derived Hepatocytes

8.1 Techniques for Deriving Hepatocytes from iPSCs and ESCs

Chistiakov et al gives a helpful summary of conventional methods that can be used to differentiate both embryonic and induced pluripotent stem cells into hepatocyte like cells (Figure 27) [66]. Figure 27 shows methods for generating hepatocytes and hepatocyte like cells from ESCs and iPSCs through spontaneous differentiation with embryoid body formation, enrichment of those bodies with definitive endoderm. Figure 27 also shows directed differentiation of stem cells with sequential deployment of growth factors that replicates the embryonic development of the liver. The specific markers expressed at different stages of directed differentiation are shown in the grey box.

Figure 27. Summary of the conventional methods for differentiating embryonic and induced pluripotent stem cells into hepatocyte like cells [66]



8.2 ReproCELL

ReproCELL is a “Pioneer in ES/iPS cell research” in Japan and deserves a lot of credit and attention for successfully producing “the world’s first commercial human iPSC-derived hepatocytes” [96]. Finding information on their differentiation protocol is difficult with both the “Publication” and “Q&A” links of their website directing to a webpage displaying “This page is under construction” [96]. ReproCELL has established a protocol they claim can produce human iPSC derived, virtually limitless number of completely differentiated, top quality hepatocytes (ReproHepato cells) for high-throughput screening with minimal batch variability [97]. According to the ReproCELL website, the ReproHepato cells have morphology and characteristics like typical human hepatocytes [97]. They also express key hepatic components like HNF4α, CYP3A4, E-cadherin and albumin in high percentages, which suggests the cells are of high purity [97]. Activity and inducibility of CYPs (3A4/1A2/2C9/2C19) is observed at levels similar to hepatocytes [97]. CYP2D6 activity can also be observed in the forthcoming version of ReproHepato (currently under development) [97]. A range of ReproHepato products specialised for analysing a wide selection of hepatic functions including Cytochrome P45 isozymes, will be launched every six months [97].

A lot of the ReproCELL scientific founders and advisors work out of Kyoto University and the University of Tokyo. The first human iPSCs were produced at Kyoto University in 2007. In 2011, a combination of Japanese universities and institutes including Kyoto University and the University of Tokyo published a paper detailing efficient generation of functional hepatocytes derived from both iPSCs and ESCs by HNF4α transduction [98]. In 2011, Takayama et al built on previous reports that hepatic differentiation of human ESCs and iPSCs was promoted by the transduction of SOX17 and HEX [98]. However the cells produced from the previous methods were only like hepatocytes and therefore were not mature enough for drug screening [98]. In this study, Takayama et al investigated the role of HNF4α in hepatic differentiation from human iPSCs and ESCs [98]. The human iPSC and ESC derived hepatoblasts were efficiently produced by the sequential transduction of SOX17 and HEX [98]. They were then transduced with HNF4α expressing adenovirus vector (Ad-HNF4α) [98]. Finally, the expression of hepatic markers of the generated cells were monitored and evaluated. This report claims that conventional methods of inducing iPSC and ESC differentiation like the stepwise procedures that use growth factors lead only to a

heterogeneous hepatocyte population unsuitable for drug screening [98]. This claim is backed up by this literature review.

Figure 28 shows the protocol for differentiation of human iPSCs and ESCs into hepatocytes via definitive endoderm cells and hepatoblasts [98]. Before the commencement of cellular differentiation, the medium of human iPSCs and ESCs was swapped for a defined serum free medium (hESF9) and cultured [98]. hESF9 consisted of hESF-GRO medium supplemented with 5 μg/ml of human apotransferrin, 10 μg/ml of human recombinant insulin, 10 μmol/l of ethanolamine, 10 μmol/l of β-mercaptoethanol, 10 μmol/l of sodium selenite, 100 ng/ml of heparin, 10 ng/ml of FGF2 and oleic acid conjugated with fatty acid free bovine serum albumin (BSA) [98].

The differentiation protocol for the induction of definitive endoderm cells started when the iPSCs and ESCs were separated into single cells and then cultured for three days on Matrigel in hESF-DIF medium with 5 μg/ml of human apotransferrin, 10 μg/ml of human recombinant insulin, 10 μmol/l of ethanolamine, 10 μmol/l of β-mercaptoethanol, 10 μmol/l of sodium selenite, 0.5 mg/ml of BSA and 100 ng/ml of activin A [98].

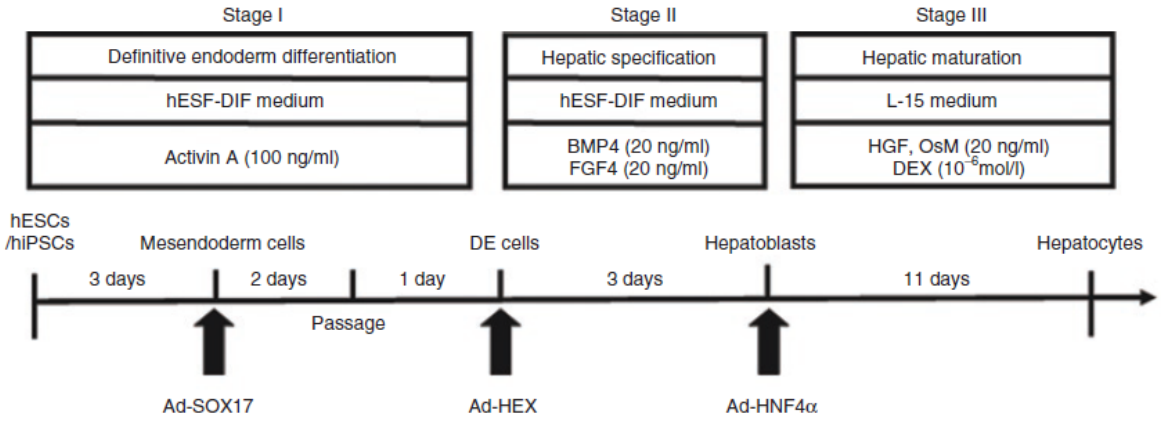
To produce mesendoderm cells and definitive endoderm cells, the derived cells were transduced with 3000 vector particles per cell of Ad-SOX17 for 1.5 hours on day three and cultured until day six on Matrigel in hESF-DIF medium with 5 μg/ml of human apotransferrin, 10 μg/ml of human recombinant insulin, 10 μmol/l of ethanolamine, 10 μmol/l of sodium selenite, 10 μmol/l of β-mercaptoethanol, 100 ng/ml of activin A and 0.5 mg/ml of BSA [98].

To initiate the induction of hepatoblasts, the definitive endoderm cells were transduced with 3000 vector particles per cell of Ad-HEX for 1.5 hours on day six and cultured for three days on a Matrigel in hESF-DIF medium with 5 μg/ml of human apotransferrin, 10 μg/ml of human recombinant insulin, 10 μmol/l of ethanolamine, 10 μmol/l of sodium selenite, 10 μmol/l of β-mercaptoethanol, 0.5 mg/ml of BSA, 20 ng/ml of FGF4 and 20 ng/ml of BMP4 [98].

During hepatic differentiation, hepatoblasts were transduced with 3000 vector particles per cell of Ad-HNF4α for 1.5 hours on day nine and were cultured for eleven days on Matrigel in L15 medium with 8.3% FBS, 8.3% tryptose phosphate broth, 10 μmol/l of

hydrocortisone 21-hemisuccinate, 25 mmol/l of NaHCO3, 1 µmol/l of insulin, 20 ng/ml of HGF, 20 ng/ml of OSM and 10^{-6} mol/l of DEX [98].

Figure 28. Schematic of the hepatic differentiation of human iPSCs and ESCs transduced with three factors. It shows the protocol for differentiation of human iPSCs and ESCs into hepatocytes via definitive endoderm cells and hepatoblasts. The hESF-DIF medium (differentiation medium for human ESCs) was supplemented with 10 µg/ml of 5 µg/ml of human apotransferrin, human recombinant insulin, 10 µmol/l of ethanolamine, 10 µmol/l of β-mercaptoethanol, 10 µmol/l of sodium selenite and 0.5 mg/ml of fatty acid free bovine serum albumin (BSA). The L15 medium was supplemented with 8.3% foetal bovine serum (FBS), 8.3% tryptose phosphate broth, 10 µmol/l of hydrocortisone 21-hemisuccinate, 25 mmol/l of NaHCO3 and 1 µmol/l of insulin. [98]



To induce hepatic maturation, transduction of the hepatocyte nuclear factor 4α (HNF4α) gene was used [98]. HNF4α is thought of as an important regulator of liver specific gene expression [98]. The transduction of Sry-related HMG box 17 (SOX17) and hematopoietically expressed homeobox (HEX) were used to induce adenovirus (Ad) vector mediated over expression of HNF4α in hepatoblasts which resulted in the upregulation of mature and epithelial hepatic markers (like cytochrome P450 enzymes) and the promotion of hepatic maturation by initiating the mesenchymal to epithelial transition [98]. Previous techniques for hepatic differentiation that used growth factors without gene transfer resulted in heterogeneous hepatocyte populations [98]. The HNF4α transduction resulted in a practically homogeneous hepatocyte population as well as the upregulation of several hepatic marker expression levels [98]. The efficacy of this hepatic differentiation technique was based on ALB, CYPs and ASGR1 expression all being around 80% [98]. HNF4α transduction upregulated the activity of several CYPs [98]. The hepatocyte like cells displayed many mature hepatocyte functions [98]. The Ad-HNF4α-transduced cells

demonstrated many features of hepatocytes including glycogen storage, LDL uptake as well as ICG (Indocyanin Green) uptake and excretion [98]. Yu et al concluded that the transcription factor HNF4α plays an essential role in the hepatic differentiation from human ESC derived hepatoblasts by initiating the mesenchymal to epithelial transition [98]. The study used both human ESCs and iPSCs. All cell lines demonstrated efficient hepatic maturation, indicating that Yu et al's technique could be used for differentiating ESCs or iPSCs into functional hepatocytes [98]. The derived cells could also catalyse the toxication of multiple compounds like primary human hepatocytes and therefore could be used to predict drug toxicity [98]. The protocol reported in this paper is the most advanced found during this literature review. It is impossible to say whether this is the exact protocol used by ReproCELL but if it is not, there is a good chance it is very similar.

9. Summary

There are not enough liver donations made each year to meet the demand created by liver disease. If BALs and hepatocyte transplantations are to treat or manage liver disease, they will require a fully functional, highly differentiated human hepatocyte line. A lot of work has been invested in stem cell research over the last twenty years and this has rapidly led to a great deal of progress. Growing up whilst a lot of these developments were going on, it seemed like the media tended to display stem cell research and therapeutic cloning in general in a negative light from Dolly the sheep to the Vacanti mouse with what looked like a human ear grown on its back. Stem cell research was depicted as mad scientists playing God. Whereas it feels like more recent stories have been reported in a much more positive way; with stem cells representing a new hope for curing many diseases including Parkinson's, multiple sclerosis and diabetes. It is worth pondering what if these hope inspiring stories had introduced the general public to stem cell research? Would there still be such strong opposition to the use of stem cells? Does the general public know there are different forms of stem cells besides ESCs?

ReproCELL were the first and today still the only commercial source of stem cell derived hepatocytes but they were also the first supplier of iPSC derived cardiomyocytes (2009) and neurons (2010). Japan seems to be constantly at the forefront of stem cell research from producing the first iPSCs in 2006 and first human iPSCs in 2007 to the work being done at ReproCELL today. Perhaps the USA would be nearer the front if not for President Bush's ban on the federal funding of embryonic stem cell research between 2001 and 2009. Hay et al and Sullivan et al at the University of Edinburgh have produced a lot of ground breaking work relating to stem cell to hepatocyte differentiation procedures. This may be due to the high occurrence of Alcoholic Liver Disease in the Scottish population especially the males.

The most impressive research found during this review was by Yu et al. The protocol Yu et al used, generated highly functional cells that can be used to mimic hepatocytes in drug toxicity testing. Yu et al's differentiation technique can be used on ESCs and iPSCs with pretty indistinguishable results. In fact no evidence was found to support any claims that ESCs or iPSCs differentiated into hepatocyte like cells with better efficiency or resulting functionality than their counterparts.

The use of embryonic stem cells requires the destruction of blastocysts formed from in vitro fertilised human eggs. If a person believes that human life begins at conception, destroying the blastocyst is considered morally unacceptable. 'People for the ethical treatment of animals' (PETA) estimate that more than 100 million animals suffer and die in experiments every year. If some of these experiments could be carried out on ESC derived disease models or drug toxicity tests would this not be better? Is a few day old human blastocyst worth more than the life of a mouse? ESCs are derived from a different person's embryo and therefore could face rejection from the recipient's own body. ESCs are also easier to culture than iPSCs in vitro because they do not require reprogramming.

iPSCs are taken from the patient's own body (autologous), so they would not be rejected by the patient's immune system. iPSC derived cells have another major advantages over their ESC counterparts and that is they do not require embryos and therefore avoid a lot of the controversy surrounding embryonic stem cells. However iPSCs still require animal testing to assess their pluripotency and therapeutic potential. If iPSCs are to be used clinically several issues must be addressed including whether using viral vectors like c-Myc (an oncogene that can cause cancer if over expressed) increase the risk of tumour formation. Due to a mutation, c-Myc is constantly expressed in many cancers and leads to an unregulated expression of many genes including those involved in cell proliferation. The retroviruses insert DNA anywhere in the genome of the cell; this could also activate the expression of cancer causing genes. In the author's opinion any increased risk of cancer far outweighs the ethical concerns surrounding embryonic stem cells and he would therefore nominate ESCs as a better cell source until a new procedure for inducing pluripotency is developed or it is proven that transplanting iPSCs instead of ESCs does not increase the risk of cancer. At present reprogramming adult stem cells into iPSCs remains too inefficient for use in everyday clinical applications.

There does not seem to be enough research focussing on MSCs considering they have shown to regenerate the liver post liver injury in mice. They have also been used clinically to treat diseases like leukaemia and non Hodgkin's lymphoma in the form of bone marrow transplants for over fifty years. There are other potential sources of MSCs including: adipose tissue, olfactory mucosa, articular cartilage, muscle and other tissues but these also require invasive procedures to retrieve them. Bone marrow transplants are very high risk but there is no reason why umbilical cords filled with vast reservoirs of MSC rich blood

could not be used. Currently umbilical cords are clamped, cut and incinerated after a baby is born. Before being incinerated, if the cords are pierced with a needle up to a quarter pint can be drained and then the blood can be cryopreserved. Just like with iPSCs there would be no issue of immune rejection with autologous MSCs if the cells were used to treat the baby when it grows up but the MSCs could face immune rejection if transplanted into another person.

Differentiating stem cells into functional hepatocytes has not yet been successful in vivo and acquiring pure populations of hepatocytes or hepatocyte like cells remains difficult. Several concerns still have to be addressed before stem cell derived hepatocytes or any other cells derived from stem cells can be successfully and safely used clinically. They must be proven stable, incapable of transmitting genetic mutations or harmful pathogens and unable to form unwanted tissues or teratocarcinomas. More research is necessary to gain a better understanding of stem cells in terms of their structure, variability, growth and utility before they can be used clinically.

The next stage of this research will probably be to introduce 3D stem cell cultures. Growing cells on flat surfaces does not accurately replicate the environment experienced by cells in vivo. 3D cultures induce stem cell proliferation by promoting cell growth and migration. 3D cell culture scaffolds offer a better representation of the in vivo environment encountered by stem cells in a living organism. Replicating these natural 3D conditions, results in more precise biochemical and physiological responses in intercellular interactions. In 3D stem cell culture, the cells would behave and react similarly to how they would react in vivo to both internal and external stimuli like variations in temperature and pH. 3D models would allow stem cells to be better examined in terms of their interactions, architecture, versatility, growth, aging and utility. A 3D culture could also increase the differentiation rate, viability and functional features of the derived hepatocytes. It could help achieve the ideal 100% directed differentiation ratio of stem cells to hepatocytes.

Looking at how many exciting developments have taken place in this field over the last ten years, if these developments continue over the next ten or twenty years stem cells will undoubtedly play a key role in cellular therapies, drug toxicity testing, support devices and drug modelling. With almost constant progress being made in this field, stem cells are a more promising source of hepatocytes than ever before.

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