Differentiation of human embryonic stem cells into hepatocytes and their in vivo application for hepatitis C viral production

by

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Abstract

Introduction: Chronic hepatitis C virus (HCV) infection is a global problem. The World Health Organization estimates that about 170 million individuals around the world are infected with HCV. Chronic HCV has a high rate of morbidity and mortality due to cirrhosis and hepatocellular carcinoma. It is a major indication for liver transplantation. The current treatment is interferon α and ribavirin of which only 50% of cases show sustained virological responses and clinical signs of improvement, indicating the need for further exploration of novel anti HCV drugs [1].

Several small animal models capable of supporting HCV infection in vivo have been achieved by the transplantation and expansion of primary human hepatocytes into the livers of mice[2]. The major limitations of these models are the generation of a supply of hepatocytes, which must come from human donors, and the technical difficulties associated with their isolation.

Human embryonic stem cells (hESC) are pluripotent cells derived from the inner cell mass of blastocytes during early embryonic life [3]. These cells are capable of self-regeneration and differentiation into any adult cell type in the human body. In the last few years, multiple centers around the world have successfully generated mature hepatocytes from human embryonic stem cells. Therefore, it is possible that hESCs can be used as a substitute for primary human hepatocytes in a small animal mouse model.

Our primary objective was to explore the possibility of differentiating hESCs into hepatocyte-like cells that could be used as substitutes for primary human hepatocytes in an SCID/UPA mouse model. As such, these studies are expected to increase the accessibility and utility of the SCID/UPA mouse model for a variety of applications, including the testing of the efficacy of antiviral strategies targeting the HCV lifecycle.

Methods: According to a published procedure[4], human embryonic stem cells underwent a multi-stage differentiation protocol to render them hepatocyte-like. The successful transition of hESCs to hepatocytes was monitored by indirect immunofluorescence detection of various protein markers at each stage of the differentiation process. The ability of the differentiated human hepatocytes to engraft and support productive HCV infection was evaluated in vivo subsequent to their transplantation to the livers of SCID/UPA mice using procedures previously established in our lab.

Results: Successful differentiation of hESCs into hepatocyte-like cells was demonstrated with indirect immunofluorescence. Thirty-five SCID/uPA mice were transplanted with undifferentiated hESCs (n=7), primary human hepatocytes (PHHs) (n=9), or differentiated human hepatocytes (DHHs) (n=19). After transplantation, serum analysis of mice from the DHH group showed undetectable levels of human alpha-1 antitrypsin (hAAT) and HCV viral production. By contrast, mice transplanted with PHH secreted hAAT values ranging from 229–1515 ng/ml, and 3 out of the 9 mice showed detectable HCV RNA levels. At the end point of the study, the mice with the transplanted liver cells were collected and examined for the presence of Alu repeat sequences using PCR and in situ hybridization techniques. DHH group showed weak signals with PCR and no evidence of histologically intergrated human cells with in situ hybridization; while histologically integrated human cells were readily detected in mice transplanted with PHH or undifferentiated hESCs.

Conclusion: Our in vivo studies showed no evidence of engraftment or HCV viral production in SCID/UPA mice transplanted with DHHs. Unfortunately; our experiments provided unsatisfactory or negative data to support the use of DHHs as a substitute for PHHs in SCID/uPA mice.

Dedication

I would like to Dedicate my thesis to

My Father
Ibrahim Mohammed Alsagheir
&
My Mother
Fatemah Almohaimed

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List of abbreviation

AFP	Alpha fetoprotein
Alb	Albumin
Alb-uPA	Albumin-urokinase-type plasminogen activator
BMP	Bone morphogenetic protein-
CCI_4	Carbon tetrachloride
CK	Cytokeratin
CYP	Cytochrome P450
Cryo	Cryopreserved human hepatocytes
Dex	Dexamethasone
DHH	Differentiated human hepatocytes
EBs	Embryoid bodies
ELISA	Enzyme-linked immunosorbent assay
ESCs	Embryonic stem cells
Fah	Fumarylacetoacetate hydrolase
HCV	Hepatitis C virus
hESC	Human embryonic stem cells
hESCs-CM	Human embryonic stem cells-derived cardiomyocytes
hAAT	Human alpha 1 antitrypsin
HGF	Hepatic growth factor

ICM	Inner cell mass
iPSC	Induced pluripotent stem cells
ISH	In situ hybridization
ITS	Insulin-transferrin-sodium selenite
MSCs	Mesenchymal stem cells
NaB	Sodium butyrate
NOD-SCID	Non-obese diabetic/sever combined immunodeficient
OSM	Oncostatin M
PCR	Polymerase chain reaction
Pdx1	Pancreatic and duodenal homeobox factor-1
PHH	Primary human hepatocytes
PI3k	Phosphatidylinositol 3-kinase
SCID/uPA	Severe combined immune deficient /urokinase-type plasminogen activator
SHH	Sonic hedgehog
TGF-β	Transforming growth factor-β

Chapter 1:

Human Embryonic Stem Cells: Overview, differentiation into hepatocytes, and applications

1.1. Background

1.1.1 Definition of stem cell

"A stem cell is most commonly known as a cell that has the ability to split to make a clone of itself and another specialized cell type" [2,5,6].

Stem cells have the ability to remain in an undifferentiated state, proliferate, or differentiate depending on both intrinsic and extrinsic factors. Not all stem cells share the same potency. Stem cells are said to be totipotent if they are able to produce all types of cells and tissues both embryonic and extra embryonic. Pluripotent stem cells, on the other hand, are able to form only embryonic cells and tissues, whereas the term "multi-potent" refers to cells able to generate several adult type cells. Lastly, cells that are only limited to producing single-typed cells are called unipotent stem cells [6-8].

1.1.2 History of stem cells

The mid 1800s marked the beginning of the discovery of stem cells. Researchers realized that certain cells could be produced from other cell types. As the 1900s proceeded, the first real stem cells capable of generating blood cells were discovered. In 1961, Till and McCulloch established the stem cell science foundation. The two Canadian scientists published their findings in *Radiation Research*, proving the existence of stem cells. Their study concluded that these cells are able to renew themselves and thus can be used in various ways [3].

One of the more popular applications of stem cell research during that time was for bone marrow transplants through the use of adult stem cells. The early part of the 1900s, show the oral administration of these bone marrow samples to patients suffering from leukemia and anemia. Of course, that therapy proved to be unproductive. However, several other experiments have since demonstrated that mice with defective bone marrow can be regenerated through infusions of bone marrow obtained from healthy mice into the bloodstream, which caused physicians to conjecture that it is indeed possible to transport bone marrow between humans. Several attempts failed before Jean Dausset was able to determine and differentiate the first human histocompatibility antigens in 1958, which was a major breakthrough that offered great potential for the use of bone marrow stem cells in various areas. These stem cells can be utilized to form different kinds of cell types and tissues. One of the areas of research was to produce functional hepatocyte-like liver cells [6,9].

Studying the early phases of embryonic development gave way to the exploration of embryonic stem cells. As part of the study of embryonic development during the late 19th century, scientists tried to prolong the viability of an early rabbit embryo in vitro. In 1890, the transmission from one womb to another of a fertilized ovum was successfully executed by Walter Heape [3,10]. Later in 1942, Nicholas and Hall reported that separation of the two blastomeres that result from the first zygote division and subsequent implantation in a different rabbits' wombs, identical twins would be born [11,12].

In 1959, the first in vitro fertilization was achieved in rabbits. Ten years later, Edwards was able to accomplish the same feat in humans [13,14]. Earlier, Edwards reported that blastomeres can be grown and divided in vitro. He also observed an inner mass of cells that was smaller in size and stuck firmly together, forming what was called an embryoid body. Embryoid bodies contain all three germ cell lines that

form all types of adult body cells, which suggests that the inner cell mass is pluripotent. Robert Edwards, Krzystof Tarkowsky, and Richard Gardner confirmed the pluripotency of the inner cell mass by reporting that if cells from the early blastocyst of a mouse embryo were isolated and implanted into a different mouse embryo's inner cell mass a chimeric mouse would be born. This is also true if these cells were harvested as early as the morula stage [10,12,15].

In the 1950s, Leroy Stevens harvested pluripotent stem cells from testicular teratomas, which became the first embryonal carcinoma cell line, which earned their name because of their similarity to early embryo cells. These cell lines could be cultured indefinitely [16], and in 1975, Martin Evans and Gail Martin showed that these embryonal carcinoma cells had the capability to differentiate into all three germ layers [17]. Different experiments confirmed such cells can divide indefinitely and are considered pluripotent. The only issue with these cell lines was that they were of a tumor origin and showed severe chromosomal abnormalities, rendering them unsuitable for future therapeutic applications in humans [6,18-20].

However, embryonic carcinoma cell lines were useful for pluripotent embryonic stem cells' appropriate culture optimizing cultural conditions and isolation techniques. This lead to the isolation of the first mouse embryonic stem cell line in 1981 (Evans and Kaufman, 1981; Martin, 1980-1981) [21]. Subsequently, there was a generation of nonhuman primate ESC lines (Thomson et al., 1995; Thomson et al., 1996) [22,23], which successfully led to Thompson and coworkers' (1998) and Reubinoff and coworkers' (2000) first generation of human embryonic stem cell lines [3,24]. While this research was able to pave the way for other studies, it also brought about ethical issues due to the fact that such stem cells were harvested by destroying human embryos.

By August 2001, President Bush prohibited all federal funding of embryonic stem cells. However, this policy did not stop the stem cell research in the private sector [25]. In 2007, President Bush encouraged scientists through an executive order to look for an alternative source for stem cells. By the end of 2007, a study was published exploring the alternatives. Shinya Yamanaka and Takahashi of Kyoto University were able to establish a procedure wherein pluripotent cells descended from skin fibroblastic cells by genetic alteration. Four transgenes were introduced to the skin cells, which transformed their phenotype into that displayed by embryonic stem cells [26,27].

The administration of Barack Obama announced the official order to carry on the research involving human embryonic stem cells. In 2010, Geron Corporation declared the commencement of the first human clinical trials using derived oligodendrocyte progenitor cells from hESCs. By November of the same year, a medicine company, Advanced Cell Technology, won federal approval to commence a multi-centre clinical trial, which focuses on human embryonic stem cell therapies for Stargardt's macular dystrophy [12,28,29].

1.1.3 Types of stem cells

Stem cells have been classified into different types through various experiments; these types are composed of umbilical cord blood stem cells, adult stem cells, fetal stem cells, induced pluripotent stem cells and embryonic stem cells,. Each differs in their origin and potency, offering various advantages and exhibiting certain limitations.

There are variations in adult stem cells. Some types are easily isolated and therefore more frequently used in clinical trials and experiments. These included bone marrow and mesenchymal stem cells. Others are harder to isolate; these are mostly organ specific. The main role of an adult stem cell is to serve as a substitute for

damaged or dead cells, enabling the body to repair or replace the lost tissue. Most organs or body parts possess intrinsic stem cells (skin, bone, bone marrow, muscle, liver, intestine), with the exception of the heart. Therefore, an adult stem cell is very specific to the body part from which it is derived [5].

Mesenchymal stem cells (MSCs) can be harvested from bone marrow, cartilage, fat, and tendons. They have a promising application in regenerative medicine either by direct differentiation into these tissues or indirectly by using different growth factors in vitro. MSCs are considered to be safe as they do not show any tumor tendency after transplantation [30].

Fetal stem cells (FSCs) are the stem cells harvested prior to or upon ten weeks of gestation. Similar to adult stem cells, FSCs are also specific to body parts and organs. However, the main advantage of fetal stem cells over adult stem cell is that they have the ability to grow rapidly. When a child is born, cord blood stem cells can be found in the umbilical cord. Such are rich in blood-forming stem cells, and so they are tissue specific. For this reason, cord blood cells are often utilized to treat blood-related conditions.

Embryonic stem cells are those harvested during the very early stages of an embryo. As such, these cells are considered to be immature and not tissue-specific, which is the main advantage of this cell line [3,31].

The last type is what is known as the induced pluripotent stem cells (iPSC). These cells can be modified in order to function as a specific cell type. These cells are considered one of the latest innovations in stem cell research; they were discovered in 2006. The cells are engineered by coaxing specialized cells (adult type cell) to express genes that are exclusively expressed in embryonic stem cells, allowing them to behave exactly as embryonic stem cells [27]. As these cells are considered to be a new discovery, further research will be needed to refine procedure and guarantee both the

effectiveness of treatment and patient safety. All types of stem cells are either multipotent or unipotent, with the exception of both embryonic stem cells and induced stem cells, which are pluripotent.

1.2 hESC and induced pluripotent stem cell (iPSC)

1.2.1 Human embryonic stem cells (hESC)

1.2.1.1 Definition

Human embryonic stem cells (hESC) or human pluripotent stem cells are usually collected from early human embryos and are characterized as being indefinitely self-replicating, dividing and producing cells that are similar to themselves. These cells are harvested from the primary layers of a human embryo and can differentiate into the ectoderm, mesoderm, and endoderm layers; these layers are responsible for forming the embryo [3,24,31].

1.2.1.2 Source

Early mammalian development consists of a fertilized oocyte. This oocyte undergoes several mitotic divisions producing from 12-32 cells collectively referred to as a morula. The morula contains totipotent cells characterized by their ability to form both embryonic and extra embryonic tissues. Five days after fertilization of the oocyte, it enters the blastocyst stage. During this stage, the embryo is made up of two layers: the trophectoderm and the inner cell mass. The latter is made up of pluripotent cells that are responsible for the formation of all tissues of the embryo [3,32]. This is where human embryonic stem cells originate. Such cells are isolated using immunosurgical[11], mechanical, and laser beam[33] techniques and are then plated onto a mouse embryonic fibroblast (MEF) feeder layer [5,24,34]. This combination results in the formation of colonies of undifferentiated cells. The colonies are then re-plated and cultures are aggregated, which allows them to differentiate into specialized cells this

time. The most common tissues derived from this procedure are cardiac, hematopoietic, neuronal, skeletal, and smooth muscle tissues (Figure 1.1).

1.2.1.3 Properties of hESC

Embryonic stem cells are unique for their unlimited ability to proliferate, if provided with suitable conditions. Their proliferative and self-renewal abilities depend on maintaining certain signal pathways and inhibiting or blocking differentiation pathways. This is achieved by high concentrations of bFGF, TGFb/Activin A/Nodal via the SMAD2/3 pathway, which plays a significant role in these processes. In addition, repression of bone morphogenetic protein (BMP) by noggin has been suggested to sustain undifferentiated proliferation of hESC in serum-free media[24,35]. ESCs are also special in the sense of possessing high telomerase activity and their preservation of a normal karyotype when cultured for a long time (34-140 passages) [18,36]. Human embryonic stem cells can also retain the ability to differentiate into the three embryonic germ layers, which later give rise to all types of body cells. This capability was discovered by in vivo teratoma formation as well as allowing an in vitro differentiation to form embryoid bodies [7,24,37,38].

Telomeres are sequences of non-coding DNA plus proteins located at the ends of linear chromosomes. These structures provide genomic stability and maintain structural integrity with successive cell divisions. These telomeres are maintained by an enzyme called telomerase, a reverse transcriptase, which insures that the replicating DNA does not shorten with repeated cell division. In most normal human somatic cells the process of consistent division and replication after birth lead to decrease of telomerase activity and shortening of telomere which eventually results in senescence, a state where the cell is no longer able to divide [39,40]. In comparison, high telomerase activity is known in embryonic stem cells sustaining telomere length and hinders the process of senescence from replicating. This process allows embryonic

stem cells to continue expressing the markers of undifferentiation even after two years of culturing [35,41,42]. Adult stem cells on the other hand are limited to a maximum of 50 divisions during culture.

1.2.1.4 Ethical issues with hESC

The use of hESCs offers much potential in alleviating diseases and injury due to the cells' ability to self-replicate. However, many are hesitant with the clinical applications, for using hESC means destroying a human embryo. Embryonic stem cells are harvested during the fifth day after fertilization of an ovum, by separating the inner cell mass of the blastocyst from the trophoblast. Once this is done, the blastocyst cannot develop further. Thus, many view this as unethical and morally corrupt, as they claim the embryo was not given the chance to survive.

Moral status is suggested as something that is granted when a being is able to have a higher-order mental capacity, which enables them to reason and make decisions [43]. Researchers argue that the embryos do not possess any moral status and thus do not have the right to life yet. Using the same line of thinking, opponents disputed that infants have no moral status and that depriving them of life, too, should be tolerable (Marquis, 2002). They also believe embryos possess a "rational nature" but cannot fully exercise it, until they are able to reason out. The difference in the capability to reason out is the result of development that each one individual acquires; it should be respected at all times. In principle, given the proper care and environment, a single somatic cell or hESC, has the ability to transform into a human being if provided proper care and environment. As such they should be given high moral status and their existence should be preserved [26,34].

Responding to this argument, researchers argue that hESC cells do not exhibit the same potential as that of the embryo. The embryo is said to have an intrinsic capability to be viable and develop into a human being without any intervention [44].

Somatic cells on the other hand do not possess the same inherent quality. It can, however, be argued that the viability of the embryo depends on the external conditions that need to be satisfied, making this process no different than the one for somatic cells.

While some may accept that embryos are not yet granted the right to life, it is still believed that they should be regarded with respect and treated with moral constraints. As Dworkin (1992) aptly puts it:

> "The life of a single human organism commands respect and protection … no matter in what form or shape, because of the complex creative investment it represents and because of our wonder at the divine or evolutionary processes that produce new lives from old ones" (p 84).

Opponents of the stem cell research claim that treating these embryos as mere research samples does not uphold respect for the embryos, but when actually considering the benefits gained and the life-altering therapies provided by using the three embryos give a new meaning to respect [45].

1.2.1.5 Advantages of hESC

The main advantage of the use of human embryonic stem cells is that it offers the plasticity of being used to form any type of cell, unlike adult stem cells. The research on these cells can contribute extensively to the area of regenerative medicine.

These cells offers a great potential in curing diseases such as schizophrenia, Alzheimer's and spinal cord injuries. Research can also lead to the development of new drugs and medicines with testing the effects of new discoveries on human subjects eradicated. The differentiated hESCs can also provide cells and tissues needed to investigate viral diseases [46-48].

Human embryonic stem cells will help increase knowledge on the mechanism of cell development and human growth. This leads to preventive treatment to

abnormal human growth. Also the use of hESC will allow scientists to study the different lineages of the body and how these lineages generally affect the cells, tissues and organs. Likewise, the manner as to how they differentiate to form a multitude of functional cells can be investigated.

These stem cells can also provide an infinite supply of cells, tissues, and organs that can help in rejuvenating different functions in the human body. The requirement of compatibility will be eradicated. The cells generated when utilized in transplantation therapies would be "universal" in nature [49-51].

1.2.1.6 Limitations of hESCs

hESCs have three main limitations. These limitations lie in 1) the ethical issues faced when harvesting such cells, 2) the potential tumor growth of such cells in vivo, and 3) the need for immunosuppression to the recipient undergoing transplantation of such cells. Further details will be discussed in the following sections.

1.2.2 Induced pluripotent stem cells (iPSC)

1.2.2.1 Definition

Induced pluripotent stem cells (iPSC) are somatic cells that have lost their quality of being tissue specific and became pluripotent. This is achieved by genetically reprogramming somatic cells. The main advantage of these stem cells is that they are created without exploiting any embryonic cells. However, these induced stem cells are comparable to embryonic cells in their ability to fashion themselves to any type of cell found in the human body.

1.2.2.2 Sources

In 2006, Takashasi and Yamanaka introduced iPSC. They uncovered 24 different transcription factors essential for maintaining pluripotent stem cells. Their initial experiment included the introduction of all 24 genes coding for the previously mentioned factors to a mouse embryo fibroblast by retroviral transduction, and generated ES cell-like morphology and function. Many trials were conducted by these two researchers to narrow the number of genes required to only four genes with a successful generation of embryonic stem cell-like cells, which are known as induced pluripotent stem cells. The isolated four genes express Oct3/4, Klf, Sox-2, and C-Myc. These four gene products are collectively referred to as Yamanaka factors [27].

Several studies have proven that iPSCs can orginate from the liver, stomach, and skin and other mature cells in the body [32,52]. Once the mature cell is isolated, it is injected with the four previously mentioned factors to aid in the transformation and reprogramming of the cell. The newly generated cell will exhibit the same properties and characteristics as an embryonic stem cell, including morphology, self-renewal, pluripotency, and gene expression [53]. This includes giving rise to the three embryonic lineages, which form the major body organs.

1.2.2.3 Advantages of iPSC

The use of iPSC is advantageous as far as bioethics is concerned for the reason that human embryos are not considered prerequisites during cell production. These cells' capability to be transformed into cells displaying the same characteristics as embryonic stem cells allowed scientists to avoid pressures associated with the different controversial methods, such as somatic nuclear transfer or what is commonly known as cloning [36].

Another main advantage of the iPSCs is that they are created from the individual's own cells, eliminating all compatibility issues and hazards by

overcoming the barriers posted by one's own immune system. Lastly, these cells are easier to create and do not require starting materials that are difficult to retrieve or obtain.

1.2.2.4 Limitations

Since these iPSCs have the same ability as ESCs to give rise to all three germ layers, they are liable to form teratomas if left undifferentiated [20]. Furthermore, iPSCs are derived from reprograming somatic cells; this process might not be always optimum, thus resulting in the omission of some essential genes that would later lead to dysfunctional differentiation. Another setback lies in the fact that iPSCs are the result of genetic manipulations of somatic cells by retroviruses. Since viruses are the vehicle that introduces the desire genes into somatic cells, they pose the risk of transmitting transgenes that later might give rise to tumors [7,54,55].

1.3 Applications of hESC

1.3.1 Advances in deriving and maintaining of hESC

Different experiments have been conducted to improve the process of deriving and maintaining hESC. One of the main advances is that human embryonic stem cells could be derived from an embryo carrying one's identical genetic material. This is achieved by the nuclear transference of one's somatic cell into an ovum and allowing it to develop, producing histocompatible hESC available for the use of the same person who provided the initial somatic cell [14,56].

The very first method of deriving hESC is through the use of mouse embryonic fibroblasts as a feeder cell and serum-containing medium [3,21,57]. This was achieved using a culture method that had already been established [8,34,58]. Scientists were concerned about using xenogenic cells as feeder cells for future human

therapeutic applications. This apprehension led to the isolation of different human fibroblasts from fetal and adult tissues, such as skin, muscle, placenta, and the uterus, which were supported the growth of hESCs and maintained their undifferentiated state, noting the different efficiency of these feeder cells [59-61].

Further studies were conducted with the intention to support the growth and differentiation of hESC in a feeder free environment. Therefore, 175 different factors and molecular components isolated from feeder cells were identified. These were then narrowed down to only 6 recombinant proteins essential for hESCs including: pigment epithelium-derived factor, plasminogen activator inhibitor, insulin-like growth factor binding proteins 2 and 7, monocyte chemoattractant protein 1, and interleukin [62]. The development of the first feeder-free culturing system occurred where hESCs were supported on matrigel-coated dishes[47]. As a sequence, Ludwig in 2006 derived the first hESC in an animal free condition [61].

1.3.2 Differentiation of hESCs

Currently, different protocols differentiate hESCs into one of the three germ cell layers; (i.e., ectoderm, mesoderm, and endoderm), and then to more specialized tissues by mimicking the intrinsic pathways of embryonic development.

The ectoderm is responsible for forming the outer epithelial tissues, such as skin, cornea, retina, and neural tissues. Culturing the hESCs in a chemically defined media activates the phosphatidylinositol 3-kinase (PI3K) pathway through high insulin concentrations, blocking the mesoendoderm differentiation and inducing expression of neuroectoderm markers, as well as blocking the TGF-B pathway, which would facilitate ectodermal specification .

Skin tissue has two main components: the epidermis and the dermis layers. Keratinocytes are responsible for forming the keratinized surface layer in the epidermis. hESCs derives keratinocytes, a potent source for skin tissue engineering,

under defining conditions when retinoic acid and bone morphological protein (BMP) are added [63]. Corneal[64] and retinal [35,65] cells also were successfully formed by hESC.

The neuroectoderm gives rise to different types of cells: neurons, astrocytes, and olgiodendrocytes. In contrast to skin differentiation, neural cells will be generated in the absence of the BMP pathway. Different substrates can help to target the tissue needed; for example, adding laminin will generate neural cells. The addition of FGF8 and sonic hedgehog (SHH) can produce dopaminergic neurons [66-68]. FGF-2, epidermal growth factor, and retinoic acid form oligodendrocytes [35].

Mesoderm cells produce skeletal, muscle, connective tissue, excretory systems, and circulatory systems; blood vessels, endothelium [69], and cardiomyocytes [70]. During embryonic development, the mesoendoderm layer can differentiate either to mesoderm or endoderm. Both Wnt and Nodal pathways are necessary to form the mesoendoderm layer. Adding BMP-4 will further differentiate these cells into mesoderm. Several specific mesodermal markers have been identified. Brachyura, a transcription factor, has been the most frequently used to confirm mesodermal differentiation [71].

The BMP, Wnt, and Nodal pathways play an important role in the different stages of cardiomyocyte differentiation by promoting cardiac commitment and enhancing the differentiation of cardiomyocytes. Also, adding ascorbic acid to serum-free media increases the efficiency of cardiomyocytes [70,72].

Both the endothelium and blood cells have a common pathway for differentiation. Various protocols have successfully differentiated hESCs into hematopoietic progenitor cells, dendritic cells, red blood cells, and platelets. hESC-derived hematopoietic progenitors can be formed by stromal co-culture and also by adding BMP-4, IL-3, IL6, granulocyte colony-stimulating factor, Flt3 ligand, and vascular endothelial growth factor A [35,69].

Other end products of the mesenchymal lineage of the mesodermal layer differentiation are the precursor cells of bone and cartilage. hESCs can differentiate into mesenchymal precursors expressing CD73, and these can further differentiate into osteoblasts and chondrocytes. Using ascorbic acid, B-glycerophosphate, and dexamethasone can induce osteoblastic differentiation. On the other hand, BMP2 has the ability to direct the differentiation of mesenchymal cells towards chondrocytes. The alteration of the biophysical environment also influences the outcome of either osteoblasts or chondrocytes [57,73].

The endoderm gives rise to the respiratory and gastrointestinal tracts, including hepatocyte [8,49,58] and islet cells [74-76]. hESC can be differentiated into endoderm by activating the TGF-B pathway, which will inhibit neuroectoderm differentiation. By activating the TGF-B and inhibiting the PI3K pathways, definitive endodermal differentiation over mesodermal differentiation is CXCR4 is a specific cell-surface chemokine receptor often used as a marker of definitive endodermal differentiation [77].

The pancreas has both endocrine and exocrine activity. By studying early embryonic development, two main pathways were observed. Activation of the retinoic acid pathway and blockage of the SHH pathway will lead to pancreatic development [74]. hESCs will differentiate into immature pancreatic cells, expressing pancreatic and duodenal homeobox factor-1 (Pdx1); these cells can then differentiate further into either endocrine or exocrine cells[78]. Neurogenin-3, a transcription factor, regulates endocrine differentiation to all four types of the Islets of Langerhans [79].

1.3.3 Applications of hESCs

Research centers have focused on hESC and its ability to differentiate into any cell type in the body and expand indefinitely. The new hESC technology offered a potential cure for many terminal or chronic diseases. Some examples of their applications are mentioned in this section.

Insulin-dependent diabetes mellitus is an autoimmune disorder that is characterized by the destruction of pancreatic insulin-producing cells known as beta cells. Diabetes is a chronic and debilitating disorder associated with high morbidity and mortality. Its management requires lifelong insulin replacement, which itself carries complications. Naturally, scientists have dedicated their efforts towards finding a cure, which solely rested on the idea of transplanting mature pancreatic beta cells to replace the destroyed pancreatic tissue. The problem with this approach was the scarcity of pancreatic cell donors. As a result, the role of hESCs was of great interest. Major efforts were driven towards differentiating pluripotent stem cells into functional pancreatic cells in vitro, and several studies have succeeded, reporting not only insulin expression but also the production of other pancreatic enzymes as well. Furthermore, transplanting hESC-derived pancreatic cells into diabetic mice regulated of serum glucose levels without the need to use exogenous insulin[76,80]. Therefore, such transplanted pancreatic cells are able to produce and regulate insulin production [80].

The second remarkable application of hESCs is in the line of the human's non-regenerating nervous system. Some disorders are marked by neuronal cell loss. For instance, in Alzheimer's disease, a disorder characterized by pronounced dementia and impairment of function, neuronal cell death results in global brain atrophy. When spinal motor nerve cells degenerate, amyotrophic lateral sclerosis manifests as a debilitating weakness, which later involves the respiratory muscles

and make this disorder fatal [35,36,81]. Another functionally limiting disorder is Parkinson's disease, a hyperkinetic motor disorder that affects the basal ganglia and, more specifically, the Substantia Nigra, leading to the destruction of dopaminergic neurons. hESCs are a substitute for the lost neurons, and they replenish the lost brain matter. A parkinsonian rat model was designed and injected with hESC-derived dopamine-producing neurons; in another study, the model was injected with neural progenitor cells. In both studies, the rat model exhibited locomotive improvement[20,82].

Moreover, hESCs offer a potential cure for congenital immunodeficiency disorders. Patients suffering from these disorders are most susceptible to life-threatening infections and display various signs of anemia, diarrhea, arthritis, and many other conditions and disorders.. Once stem cells are put into action, they restore the immune function of these patients, dramatically improving their quality of life [83].

Another utilization of these extraordinary cells is apparent in alleviating the limitations set by bone and cartilage disorders, especially the degenerative ones, such as osteogenesis imperfecta, a disorder of collagen structure characterized by bone fragility and extensive fractures. hESCs are introduced to injured areas, repairing whatever damaged tissue is found [84,85].

Other studies focused on the applications of hESCs on animal models expressing human diseases. In one experiment, hESC-derived cardiomyocytes were injected into a pig model with slow cardiac electrochemical function; the normal, proper heart rate was restored [86]. Thus the possibility of stem cells renewing the conducting system of the heart muscle and acting as a pacemaker was considered. In addition, pluripotent stem cells were used to replace an infarcted heart muscle. This is demonstrated by the work of the Gepstein group, which used undifferentiated

hESCs and hESC-derived cardiomyocytes (hESCs-CM) on an infarcted heart model[87]. The study reported a significant preservation of left ventricular function with the application of hESC-CM without teratoma formation. In contrast, the undifferentiated hESCs generated teratomas [19,86].

Another group known as the Keirstead's research group transplanted hESC-derived oligodendrocytes and undifferentiated hESCs in a rat model. These rat models varied regarding the time the animals received the transplantation. Some were transplanted early, 10 days after the spinal cord injury, and others were transplanted after 10 months of the induced injury. This study was able to promote improvement in motor function and apparent remyelination in the group of rats that received the stem cell transplants early after the injury. Also, it was noticed that the undifferentiated stem cells resulted in teratomas whereas the hESC-derived oligodendrocytes did not [88].

Another area of research was to generate different disease-specific hESC lines. These stem cells replicate, producing disease models more feasible for researchers to study and investigate. Amongst the disease-specific stem cells were stem cells carrying the genetic mutation of the following disorders: Fanconi's anemia, cystic fibrosis, Huntington's chorea, Duchenne muscular dystrophy, and others [35].

1.4 hESC differentiation into hepatocytes

1.4.1 Anatomy and physiology of the liver

The liver is the largest internal organ in the human body, weighing 1200 to 1600 grams and located in the right upper quadrant of the abdomen just below the right hemi-diaphragm. The liver made up of the right and left lobes. The right lobe is the larger, and it is further split into two more lobes, the caudate and quadrate. The falciform ligament serves as the demarcation line between the lobes' right and left parts. This is the ligament that also attaches the liver to the anterior abdominal wall. The ligamentum teres, extends down the free edge of the falciform ligament. In contrast, the coronary ligament is the one that branches from the falciform ligament and extends over the right and left lobes' superior surfaces. This ligament connects the liver to the inferior surface of the diaphragm [89,90]5.

Glisson's capsule is the fibroelastic capsule that covers the liver. It contains the nerves, lymphatics and blood vessels that supply the liver. When a disease causes liver congestion or swelling, the distention of this capsule produces pain [89].

A large blood supply is necessary for the liver to function metabolically. It is characterized by a complex vascular system receiving blood from both arteries and veins. The hepatic artery, branch of the abdominal aorta, provides oxygenated blood, supplying blood at a rate of approximately 450 ml/min, about 25% of the total systemic circulation. The portal vein accepts the deoxygenated blood from the intestinal tract, which can be traced back from the inferior and superior mesenteric veins. This vein can deliver approximately 1100 ml/min to the liver. The blood carried in the portal vein is rich in oxygen and nutrients from the digestive tract [89,90].

The liver is a magnificent organ. It has many functions in the human body, including utilizing food in order to produce the chemicals and nutrients that maintain hemostasis. It is also the organ that processes drugs and modifies them after

absorption and serves as a storage organ for nutrients that later could be utilized when needed, such as sugars, vitamins, and minerals.

The liver also has a synthetic function, being able to produce proteins, which are the building blocks essential for tissue function and restoration. It also has the ability to neutralize poisons and metabolize alcohol. The liver is also responsible for regulating different processes such as fat transport, blood clotting activities, levels of drugs and chemicals in the blood, and maintaining hormonal balance. Furthermore, the liver synthesizes bile that aids in digestion and elimination of excess cholesterol[91].

Liver development

Early in embryonic life, the foregut forms by invagination of the endoderm. The ventral surface of the posterior foregut is located adjacent to the developing heart. Receiving signals from the heart will induce the hepatic fate and form the outer pouch, known as the hepatic diverticulum or liver bud. The hepatic diverticulum will grow rapidly and give rise to hepatoblasts. The hepatoblasts invade the septum transversum mesenchyme: a structure located near the fetal heart. Liver maturation is not completed until after birth [90,92].

1.4.2 Different approaches to differentiate hESCs to hepatocytes *in vitro*

Liver cells can be generated from latent cells produced by embryonic stem cells (ESCs). Such ESCs are considered pluripotent with self-renewing ability isolated from the inner cell mass (ICM) of the blastocyst that typically articulate Oct4, SSEA-4, TRA-1-60, and TRA-1-8. A high telomerase activity level [75,92] is also observed in ESCs, which propagate extensively and rapidly in vitro. Moreover, they can also easily be demarcated into all the three germ layers' derivatives. The embryoid bodies (EBs) known to cumulate into spheroid clumps of cells translates to natural cell demarcation with the characteristics of the three germ layers (i.e. ectoderm, mesoderm, and endoderm) [17]. The manufacture of tissue-specific cells from the ESCs

of both rodents and humans has been advanced due to the development of different protocols [83,93].

Caution must be taken when analyzing and evaluating research materials that recount the degree of mature liver cells being effectively taken from embryonic stem cells. A number of techniques have been utilized to prove that albumin and urea are secreted when harvesting stem cell-derived "hepatocytes." In addition, they display cytochrome P450 (CYP) enzyme activity. However, whether such cells can efficiently mimic the function of primary hepatocytes [50,94,95] can only be determined through a careful evaluation of their growth potential, metabolic activity, secretory function, and gene expression .

Different strategies are being used to differentiate endoderm to hepatocytes in vitro. These include 1) the reconstruction of an in vivo cell matrix as well as cell-cell interaction, 2) the addition of soluble medium factors, and 3) the determination of a cell's fate by utilizing genetic modification.

The reconstruction of an in vivo cell matrix involves the replication of the ontogenic scaffold, specifically collagen [52,96]. Apart from this, co-culture with hepatic and non-hepatic cells was added to improve the in vitro environment and encourage hepatogenic differentiation [47,62,97,98]. One limitation of this strategy is that such a technique may result in cell fusion. When this happens, a more sophisticated technique might be necessary to separate the fused cells. One way to prevent this outcome is to make use of a semipermeable membrane or a filtered cell-conditioned media [99,100]. Fetal liver cells are the most suitable cells for co-culturing hESCs, as they contribute to the hepatocellular functionality that is evident in embryonic stem cell cultures.

Another approach that is being utilized in the differentiation process is the use of growth factors and cytokines to promote the development of hepatic growth in vitro. Hormones and steroids also support this mechanism. Activin A augments cell culture, specifically definite endodermal populations[101]. The stepwise addition of these growth factors with a mixture of insulin-transferrin-sodium selenite (ITS), dexamethasone, and OSM seems to be successful in promoting functional maturation[102].

Genetic modification is driven by an identical series of transcriptional events that is evident in early liver organogenesis in vivo. This hepatic gene expression is related to HNF3β levels in a straightforward way[103]. The main limitation of this technique however, is that it is unpredictable.

Modifying the fate of the cell by direct interference with its chromatin structure was introduced a few years ago. The cell was exposed to 5mM sodium butyrate. This led to the increase in the harvest of pure hepatic cells by 10-15% and was later modified by the addition of Activin A with a smaller concentration of sodium butyrate. This modification led to a 10-70% enrichment. Also, recent studies were able to pinpoint the factors that affected the attainment of hepatic functionality. This was done through epigenetic modification as well as a stepwise exposure to stimuli, mainly cytokines [58].

Different approaches have induced ESCs to separate into "hepatic-like" cells. Hepatocyte markers, in addition, are being displayed by the liver and include the inducible expression of cytochrome P450, AFP, Alb, and CK18. There have also been reports morphologic characteristics of epithelial phenotype[8].

However, more mature hepatocyte gene expression is needed for stem cell-derived hepatocytes to be deemed useful. The cells also need to display sufficient

levels of expression similar to the levels found in normal liver cells. In addition, the differentiation has to be lineage-specific[104].

It is imperative that protein production be shown to support authentication of gene expression studies. Definitive verifications of mature hepatic function is being offered by the metabolic activity of CYP enzymes. The only albumin-producing cells are hepatocytes. However, not every albumin-producing cell is considered a hepatocyte. There are many stem cell-derived "hepatocytes" that show albumin, but a large number of them do not display the necessary genes that would comprise normal hepatocyte function. This is also true for CYP enzyme activity not limited to hepatocytes. Inducible CYP activity is nonetheless applicable to lung, intestinal, and epithelial cells and to adipose tissue as well[104].

To satisfactorily appraise the success of in vitro differentiation of stem cells, it will be imperative to noticeably display cellular distinctiveness by displayed primary hepatocytes. The comparison of gene expression by differentiated "hepatocyte-like" cells and human fetal and/or mature liver cells[91] is nonetheless necessary. There should be confirmation of basal and inducible CYP450 isoform function [105].

Also, it is necessary to determine whether hormones, ammonia and other endogenous substances are able to metabolize[91]. The synthesis as well as the production of subsequent substances must be evaluated: clotting factors, complement, transporter proteins, albumin, bile acids, lipids, and lipoproteins. Further study is nonetheless required to prove the corresponding evidence of the restoration of liver function in appropriate animal models or evidence showing the repopulation of the liver by derived "hepatocytes".

Through the usage of both gene expression and function, derived hepatocytes must be able to reveal drug metabolism and detoxification activity. They need to express hepatic transport proteins and at the same time show that transcription factors

are present in their mature form. To some level, they should also be able to secrete albumin, generate bile acids, conjugate bilirubin, and process ammonia, and also after transplantation, they must be able to function in animal models displaying liver injuries. And lastly, the differentiated cells should only express transcription factors and genes distinctive of liver cells alone[104].

1.4.3 Directed differentiation of hepatocytes

In early embryonic development, many growth factors initiate several signaling pathways that lead to the expression of transcriptional genes responsible for regulation of hepatogenesis. By understanding these molecular mechanisms and applying them *in vitro*, a human-like hepatocyte will be generated. During the past few years, various step-wise differentiation protocols have provided successful techniques to generate human-like hepatocytes in vitro by mimicking early embryonic development, with different efficiency and functional maturity.

For differentiation of hESC to definitive endoderm, both Wnt/B-catenin and TGF-B are important signaling pathways. Kubo et al. (2004) states that Activin A, a member of the TGF- superfamily, which induces endoderm by mimicking Nodal, a key component in inducing endoderm in early embryo life. Other factors also have an additive efficiency, such as WNT 3a, a member of the WNT/WG family, noggin, a BMP inhibitor, and FGF2/4 [106-108].

Several growth factors have been noticed in early embryonic development to regulate hepatogenesis. By adding FGF2 and bone morphogenic protein (BMP4) in vitro, the same cytokines produced by adjacent cardiac mesoderm and septum transversum mesenchyme, respectively, leads to the formation of early hepatoblasts. Hepatoblasts have a bi-potent capability, meaning that they are able to generate either hepatocytes or cholangiocytes [93,109].

The addition of different soluble factors, such as oncostatin M (OSM), glucocorticoid, and hepatic growth factor (HGF), promotes mature hepatic gene expression. The wnt/B-catenin pathway is involved in hepatocyte differentiation and proliferation. FGF 10 also may interact with this pathway to promote hepatocyte proliferation [93]. OSM is a member of the IL6 family, which activates the STAT2 and Ras pathways, two alternative pathways for hepatic maturation [103,110]. Glucocorticoids help maintain the differentiated hepatocyte phenotype and expression of late hepatic genes[111]. HGF is a general hepatotrophic cytokine that favors hepatic differentiation over cholangiocyte differentiation. Other factors have been observed to regulate hepatocyte maturation, such as FGF4, FGF8, EGF, follistatin-288 isoform, and retinoic acid[112,113].

1.4.4 Differentiation signaling pathways

1.4.4.1 Wnt/β-Catenin pathway

This pathway fundamentally controls the outcome of the cell during the development of an organism (Figure 1.2). The Wnt ligand is a secreted glycoprotein that attaches itself to hESC Frizzled receptors. This, in turn, produces a surge of signals that lead to the disarticulation of the multifunctional kinase GSK-3β from the APC/Axin/GSK-3β complex[114]. When the Wnt signal is not present (off-state), β-catenin, a vital cell–cell adhesion adaptor protein that also acts as a transcriptional co-regulator, is degraded through the activation of the APC/Axin/GSK-3β complex[96]. Sufficient phosphorylation of β-catenin by the synchronized action of CK1 and GSK-3β directs its ubiquitination and proteasomal disintegration *via* the β-TrCP/SKP complex[115,116].

When Wnt binding is present (on-state), phosphorylation and poly-ubiquitination of Dishevelled (Dvl) will inhibit GSK-3β, resulting in the stabilization of the

APC/Axin/GSK-3β complex and maintaining the β-catenin and Rac1-dependent nuclear levels. This allows β-catenin to attract to the LEF/TCF DNA-binding factors in the nucleus, where it will act as a transcription activator by dislodgment of Groucho-HDAC co-repressors [114]. Disruption of this pathway, exemplified by APC and Axin mutations, has been implicated in human cancers and is known to be evident in some tumors[117]. For the duration of the development process, the Wnt/β-catenin pathway combines signals from various pathways, including BMP, retinoic acid, FGF and TGF-β.

1.4.4.2 Notch Pathway

Notch signaling regulates the determination of cell-fate during stem cell maturation. The Notch pathway intercedes in juxtacrine signalling between neighbouring cells regulates the developmental outcome in neuronal, cardiac, immune and endocrine cells (Figure 1.3). The receptor of this pathway involves a single-pass trans-membrane protein made up of useful extracellular (NECD), transmembrane (TM) and intracellular domains. The endoplasmic reticulum (ER) and Golgi apparatus of the activated cell process and cleave the Notch receptors to bring about a glycosylated, calcium-stabilized heterodimer. These heterodimers are made up of NECD that is non-covalently bound to the TM-NICD expressed on the plasma membrane, known as S1 cleavage, which will facilitate ligand attachment.

In mammals, components of the Delta-like (DLL1, DLL3, DLL4) and the Jagged (JAG1, JAG2) families, positioned in the neighbouring cells (signal-sending cells), act as ligands for Notch signalling receptors. Once the ligand on the signal cells has already formed a bond, the NECD is unbound from the TM-NICD structure (S2 cleavage) *via* the enzyme TACE, where the end bracket is located (ADAM

metalloprotease TNF-α converting enzyme)[118]. In contrast, the NICD remains bound to the ligand. The newly formed complex (NICD+ligand) can now proceed to endocytosis. This also permits recycling/degradation surrounded by the signal-sending cell. Within this process, γ-secretase discharges the NICD from the transmembrane (S3/4 cleavage)[119].

The next step involved is transfer to the nucleus. This is where NICD binds with the CSL (CBF1, RBPJK) family, allowing the transcription process to proceed[120]. As a result, canonical Notch target genes Myc and HES-family members are activated. Significantly, the notch pathway dysregulation is known to be involved in several disorders. Mutations that lead to activation of this pathway and the accumulation of NICD are known to be implicated in adult T-cell acute lymphoblastic leukemia and lymphoma [121]. Likewise, Notch receptor and ligand mutations resulting in inhibition of this pathway are also implicated in other disorders[122]. Understanding this signalling pathway gave rise to potential pharmacological intervention.

1.4.4.3 Transforming growth factor-β (TGF-β)

The transforming growth factor-β (TGF-β) superfamily signals pathway functions in the regulation of cell development, demarcation and maturity in a broad array of organogenesis. The TGF-β superfamily contains more than 40 factors, including activin A, inhibins, anti-mullerian hormone, BMP and TGF-β[38,123]. The TGF-β signalling pathway is triggered by the ligand-induced oligomerization of the heterodimer and activation of serine/threonine receptor kinases. This leads to phosphorylation of Smad2 and Smad3 molecules in the cytoplasm (Figure 1.4). In contrast, in the bone morphogenetic protein (BMP) pathway, Smad 1/5/8 is utilized. The carboxy-terminal phosphorylation of Smad 2 and 3 attracts Smad4, leading to the development of the Samad complex and translocation to the nucleus[94,124,125]. The

different biological effects are then regulated by the activated Smads *via* inducing transcription of the targeted genes. Attenuation of the activin and BMP pathways is conducted by MAPK signaling at various levels[94,116]. Activation of Smads (I-Smads) 6 and 7 is stimulated by activation of both the activin/TGF-β and BMP signalling pathways, which act as a negative feedback and inhibit this pathway.

1.4.5 Direct differentiation in *in vivo* settings:

Transplantation is one of the main goals for the differentiation of hESCs into hepatocytes; however, only a few studies have evaluated this or reported findings. Transplantation of hESC-derived hepatocytes can address the following two questions: Can these cells further mature *in vivo*? Are the cells functional? Moreover, this will provide a more accessible way to compare differentiated cells with primary human hepatocytes.

One of the earlier studies *in vivo* was reported by Cai et al. (2007) [8]. They differentiated hESCs into DHH cells using three stepwise approaches, and then transplanted the differentiated cells into the carbon tetrachloride (CCI_4)-injured livers of non-obese diabetic/severe combined immunodeficient (NOD-SCID) mice through intrasplenic injection. Two months later, the mice showed some evidence of chimerism evidenced by a few human alpha 1 antitrypsin (hAAT)-positive cells. They suggested that their low-efficiency engraftment was due to the use of CCl_4-injured NOD/SCID mice [8].

Agarwal et al. (2008) reported transplantation of definitive endoderm to explore whether these cells could differentiate into hepatocytes in an *in vivo* environment . They differentiated hESCs into definitive endoderm using activin A, then transplanted the cells into the CCl_4-injured livers of NOD-SCID mice through

intrasplenic injection. Four weeks later, human liver cells were detected at a low level in <1% of hAAT and CD26. Provided that these cells could further mature *in vivo* [126].

Touboul et al. (2010) transplanted hepatocytes derived from hESCs into 5-day-old uPAAxRag2gammac-/- mice. Blood serum samples were gathered 8 weeks post-transplantation, and human albumin secretion was found to be low, at ~3 ng/mL. Nevertheless, histological examination revealed cells expressing human albumin and hAAT in small and large clusters throughout the liver, showing the ability of these cells to proliferate in an *in vivo* environment [127].

Another approach differentiated hESCs into embryoid bodies, then further differentiated them into hepatocytes using specific growth factors and cytokines. Two studies used this technique and reported *in vivo* results. Duan et al. (2007) reported success in transplanting hESC-derived hepatocytes into NOD-SCID mice by transuding a triple-fusion protein viral vector. They concluded that these cells were engraftable and functional by expressing albumin and AAT and not expressing alpha fetoprotein [128]. Basma et al. (2010) transplanted hESC-derived hepatocytes into NOD-SCID mice that had undergone a partial hepatectomy. Mouse serum, analysed by enzyme-linked immunosorbent assay (ELISA), contained 500–1,000 ng/mL and 100–200 ng/mL of human albumin and hAAT, respectively, at 3 weeks. However, gross and histological examination showed evidence of teratomas in the liver, spleen and peritoneal cavity. To eliminate undifferentiated and/or poorly differentiated cells, the differentiated cells were sorted for the presence of asialoglycoprotein receptor, a specific marker for mature hepatocytes, and then transplanted into the spleen of albumin-urokinase-type plasminogen activator (Alb-uPA/SCID) mice. Seventy-five days post-transplantation, mouse serum contained 1,000–2,000 ng/mL of human

albumin and hAAT. Histology and gross examination showed no evidence of teratomas[129].

1.4.6 Hepatocytes derived from human embryonic stem cells and their application

Being able to produce a consistent and homogenous resource for human hepatocytes from hESCs would be a valuable tool for liver tissue engineering. These human-like hepatocytes can be used in cells based on *in vitro* assays studying metabolic profiling, drug–drug interactions, drug toxicity, viral studies or in cell therapy and regenerative medicine [75]. Hepatocyte-derived stem cells have many applications, which will be further detailed below.

1.4.6.1 Cell Therapy

The worldwide scarcity of organ donors is expected to escalate over the next few decades. As such, research on alternative techniques for curing severe liver diseases will become more and more essential. Cell transplantation, as well as other cell-based therapies, offers patients with liver failure a promising potential to lead better lives after recuperation [50,94]. The development of novel cell therapies can be anticipated once the supply of stem cell–derived hepatocytes can be increased.

It has been demonstrated that the transplantation of a few allogeneic donor hepatocytes can successfully substitute for and generate new livers in mice. Most importantly, scientists were able to grow human hepatocytes inside a liver-diseased rodent model. Following transplantation, significant levels of liver repopulation were achieved in the harshly immunocompromised mouse, which suffered from hereditary tyrosinemia, a condition characterized by fumarylacetoacetate hydrolase (Fah) deficiency. These newly repopulated livers were harvested and successfully re-transplanted into immunodeficient Fah knockout mice repeatedly over four

subsequent generations [130]. It is known that hepatocytes could be derived from an extra-hepatic stem cell resource such as the bone marrow. Mouse bone marrow–derived stem cell hepatocytes were used to repopulate Fah knockout mouse livers [9]. However, further evidence and data are lacking concerning how efficiently these stem cells can function as the primary hepatocytes [130]. A number of *in vivo* stem cell studies were conducted with various outcomes. The difference in the results may be explained by the processes each of these studies employed to implant the stem cells into the liver, the type of animal model and the form of liver injury.

The risk of developing tumors coupled with the medical use of differentiated hESCs or adult stem cells cannot be ignored. After transplantation, embryonic stem cells are known to form teratomas [38]. However, further studies regarding transplantation risk and teratoma formation of immature liver progenitor cells, or even matured stem cell–derived hepatocytes, need to be conducted. Another obstacle facing hESC techniques is graft rejection. The progress in the field of autologous stem cell transplantation, whether inducible pluripotent stem cells or adult stem cell–derived hepatocytes, may resolve the issue of rejection and the necessity of using the immune suppressive medications. Since long-term immunosuppression is associated with the development of certain cancers, the extent of caution is valid and may thus be prohibitive. These concerns need to be meticulously addressed [94,125].

The successful scaling-up of the manufacture of differentiated cells is an area that requires further examination. The procedure for increasing hepatocyte-derived hESCs may result in curtailed differentiation or compromised stem cell reliability. Primary hepatocytes are challenging to maintain *in vitro*, in the sense that they tend to de-differentiate and lose their functional features during the culturing process [94,131,132]. Thus, major modifications to the culturing processes to develop better cell-homing capacity are required.

Techniques will need to be enhanced to develop more functional ESC-differentiated hepatocytes and to prevent rejection before proceeding into human trials. One of the challenges for transplant success involves detecting the engrafted cells in recipient liver [132,133]. Since these cells may engraft sporadically, sectional biopsies and histological analyses may be rendered unfeasible unless the donor cells develop the ability to repopulate the entire liver.

1.4.6.2 Tissue engineering and drug discovery

Updates in the drug discovery process can potentially be executed by stem cell technology through the improvement of metabolic profiling, primary screening and toxicity evaluations necessary to optimize drug candidate selection[134]. Related studies have already been conducted—such as those involving liver tissue engineering, tumor cell lines, and cells immortalized by genetic transformation—but with limited success. Cell lines usually have a modified extracellular signalling system and an abnormal karyotype [91,135]. Culture and engineering studies involving primary cells may be more enlightening; nonetheless, they remain plagued by practical difficulties. Primary cells are typically populated *in vitro* using enriched media containing mitogens. After isolation of the primary cells and entrance in the cell cycle, they tend to survive only for few generations before they start to lose their hepatocyte potential. Their restricted ability to maintain hepatic features and their limited expansion potential are the main challenges involved in the utilization of primary liver cells in the *in vitro* environment, thereby disqualifying these cells for drug discovery in large-scale studies [91,136,137].

Embryonic stem cell technology presents a chance to widen the use of functionally differentiated hepatocytes for genetic analysis and drug discovery in vitro. If consistent reproducible functional derived hepatocytes from ESCs can be fashioned, the role of human genetic variation will more conveniently elaborated in

response to drugs. Likewise, by obtaining ESCs of recognized abnormal genotypes *in vitro*, the feasibility of inspecting the mechanisms responsible for the genetic predisposition to a specific liver disease would be enhanced.

The availability of derived human hepatocytes from ESCs may also influence the development of bioartificial liver devices. The progress of these devices has also been stalled by the inadequate supply of primary human hepatocytes. By utilizing tissue-engineering technology, the microstructure of the liver was examined to mimic the continuance of signalling pathways comparable to the intact hepatocyte microenvironment in order to advance *in vitro* culturing. Successful alterations have involved exploitation of the extracellular matrix environment [137], modifications in the conformation of culture media [94], and other techniques that encourage cell-cell interactions and signalling [99]. Recently, there has been a substantial development in understanding the hepatocyte signalling pathways, differentiation and function through advances in microfabrication and bioimaging technologies. These advances have allowed microscale manipulation and assessment of the cellular microenvironment *in vitro* [138,139].

Such developments have offered a new prospective modality in the differentiation procedure and the formation of liver tissue samples that more accurately resemble the physiology and pathology of human hepatocytes. Combining techniques from cellular, molecular and liver developmental biology along with tissue engineering and micromechanical schemes will generate novel systems that should make possible the invention of liver on-chip devices. Such progress can be put to an array of uses, such as the study of the hepatotoxic consequences of both drugs and environmental factors, as well as monitoring for prospective growth factors and offering programmed discharge of specific growth factors to augment hepatic stem cell differentiation [57,140].

Hepatocytes are fundamental targets for the testing of novel drugs' pharmacokinetics[131]. Furthermore, these cells can be employed for the examination of drug compounds directed towards the therapy of metabolic diseases and their subsequent effects, in addition to accessing hepatotoxicity trials. During the early stages of assessment, most of pharmaceutical companies' funding is utilized for the detection of the drugs' central metabolic and excretion pathways[8,141]. Early metabolic testing considers the accomplishment of metabolic disintegration of the compound, the mechanism of its metabolism and its interactions with drug-metabolizing enzymes.

In drug metabolism studies, hepatocytes are presently used as the gold standard[131]. There is a large demand for *in vitro* models of hepatocytes, particularly for toxicity studies of new drugs. This demand is due to the observation that withdrawal symptoms induced by experimental drugs investigated in different pharmaceutical projects are caused by the unpredictable metabolism in humans. It is foreseen that with the extensive use of hESC-derived hepatocytes in toxicology, hepatotoxicity will be the leading cause of preclinical failure of new drugs. As such, during the early drug developmental stages, new and enhanced cell models are desirable for assessing hepatotoxic effects. In many cases, toxicity is observed during the late phases of drug discovery. Hence, the species differences and extensive use of animal models in drug toxicity trials poses the main problem. To surmount this issue, consistency of the manufacturing of functional human hepatocytes is called for.

In principle, no cell type in existence today is able to completely duplicate the complexity and function of the liver. The accessible human models are isolated from either cancer-derived cell lines or from hepatocytes obtained through liver biopsies. Unfortunately, these two cell types provide essential limitations. Existing hepatic cell models exhibit very low quantities of essential enzymes and hold considerably

dissimilar levels of other key proteins in comparison to native hepatocytes [133,142]. One of the most frequently used cell lines is human hepatocellular carcinoma (HepG2), which shows poor phenotypic and functional activity compared to *in vivo* hepatocytes[133]. Human primary isolated hepatocytes could be used as a therapy in this situation. Moreover, although primary human liver cells may exist, they promptly lose their functional ability when maintained *in vitro*. A key limitation in this case is the prerequisite of repetitive sourcing [142]. The changeability and lack of clarity in these materials result in inconsistent test outcomes, and therefore, in inconvenient constraints that limit utility.

A similar model based on human cells is of prime importance. The challenge at this point is to adapt hESC-derived hepatocytes to a format for drug discovery trials. Suitable sources of hepatocytes could considerably advance the growth of new drug innovation strategies and make *in vitro* trials possible. Soto-Gutierrez (2006) first descried the metabolizing effect of drugs in cell-derived hepatic cells from hESCs[95]. The study showed that the cells significantly metabolized lidocaine. According to Soderdahl (2007), hepatocyte-derived cells from hESCs display quite a few mature liver markers[136]. Moreover, glutathione transferase activity was detected at quantities equivalent to those in primary cells. The existence of specific biotransforming enzymes is fundamental for the prospective study of stem cell–derived hepatocytes. The cytochromes and glucuronosyltransferases are the most significant drug metabolism enzymes. The liver is characterised by the complexity of encompassing cell types beyond hepatocytes that maybe necessary for the liver's drug metabolizing properties (*e.g.*, Kupffer cells, stellate cells, cholangiocytes). For this reason, to continue studying new drugs in *in vitro* settings, intricate multifaceted models need to be engineered. This additionally highlights the potential for hESCs as a resource for toxicity trials that is completely appreciable and will foresee the

positive and negative effects sooner. Optimistically, hESC studies may be able to duplicate simple liver tissue and further develop the probability of specifically predicting human toxicity *in vitro*. It is feasible that pharmaceutical corporations that effectively integrate stem cell technologies will lead in the field of drug breakthrough and discovery [25,91].

1.4.6.3 Regenerative medicine

Stem cell differentiation into hepatocytes is of vast importance. The availability of these cells in large number would permit their use as a substitute for whole organ transplantation in certain cases, and as a prospective treatment modality for end-stage liver diseases. Facing an increasing global population of hepatic patients whose care necessitates extensive economic and healthcare resources, hESCs are a potential source for producing unlimited amounts of hepatocytes needed for transplantation. There are several candidate cell types that have been considered, but hESCs are the ones that hold the highest potential due to their pluripotency and appropriateness for cell-replacement therapy. Therefore, comprehensive study and knowledge of the embryogenesis and the development of a specific cell's fate will facilitate the process of *in vitro* differentiation and artificial cell and tissue production [28,138].

1.4.6.4 Limitations

Many differentiation guidelines have already been recognized to produce almost all cell types from hESCs. Conjecturally, hESCs have unlimited application for the treatment of human diseases. The success of the developmental *in vitro* and *in vivo* models will facilitate using hESC-derived cells as a source in the regenerative field. However, many experimental obstacles must be resolved before specified cell types originating from hESCs can be applied to humans; the most crucial is a deep

understanding of the genetic and epigenetic alterations that take place during the differentiation process *in vitro*. Isolation of specific cell types that are appropriate for cell-based therapies will be indispensable. It will also be essential to control karyotypic changes during passaging. Once the derived cells are integrated into organs, they should function similarly to the native cells. Donor/recipient immunocompatibility and the lack of tumor formation must be ensured. Prior to therapeutic application, any ESC-based management should display a limited potential for toxicity, immunological rejection and tumor formation. These factors are still limitations for human stem cell applications.

1.5 Figures and tables

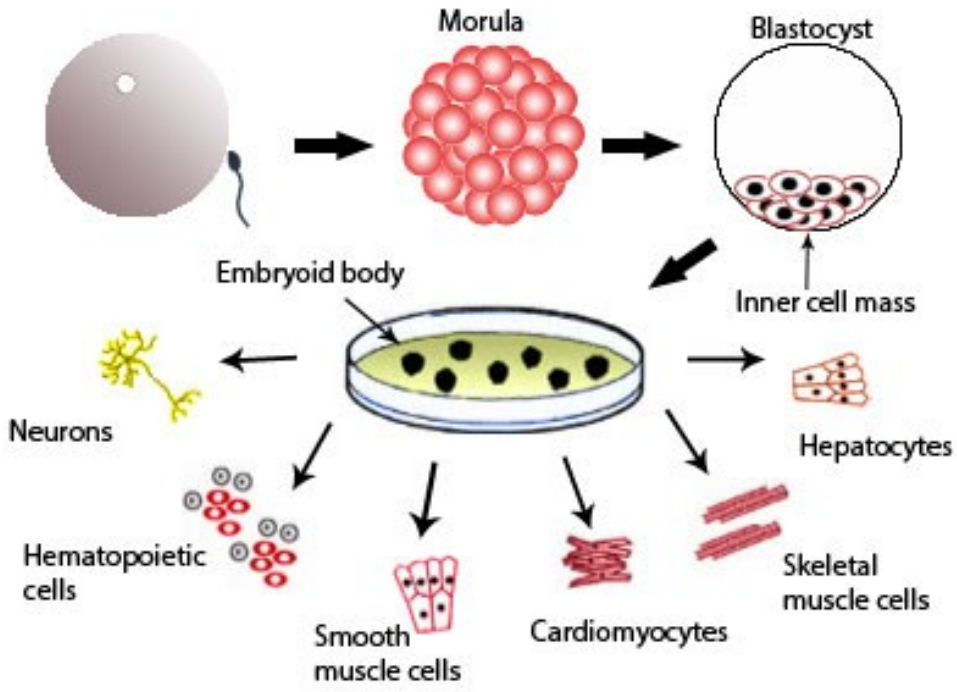

Figure 1-1 Source of human embryonic stem cells

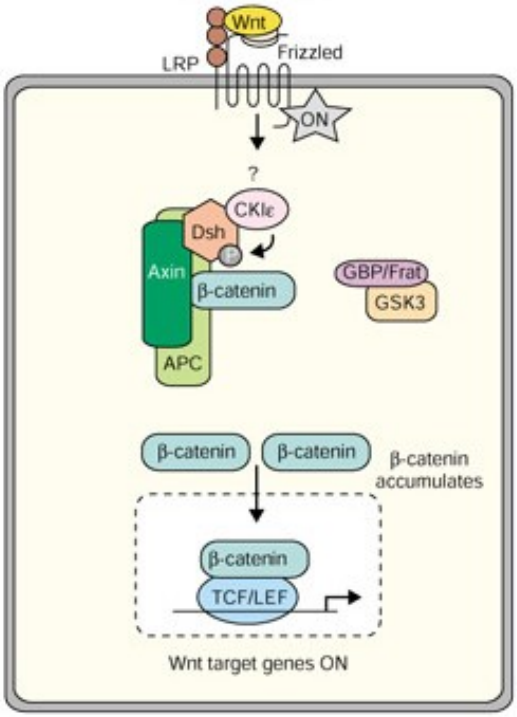

Figure 1-2 Wnt/ β-catenin pathway [143]

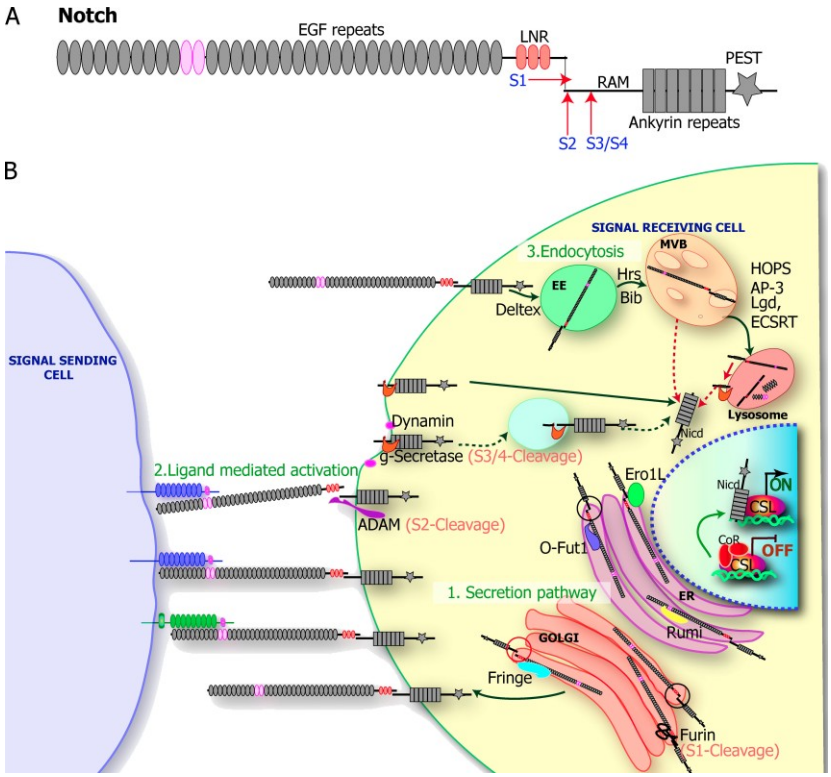

Figure 1-3 Notch Pathway [122]

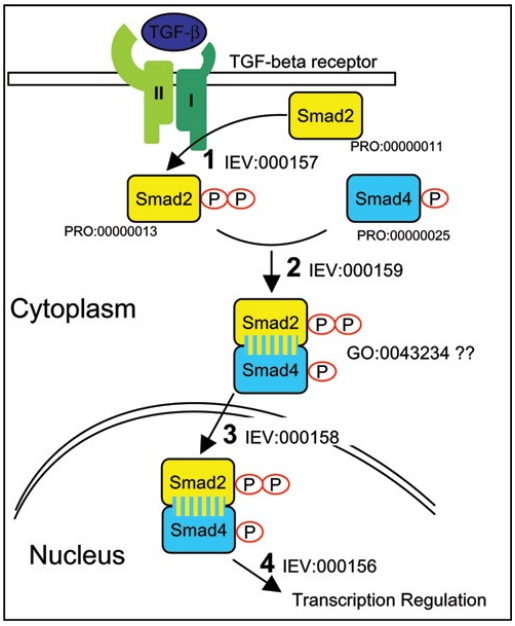

Figure 1-4 Transforming growth factor-β (TGF-β) Pathway [144]

Table 1-1 Direct Differentiation of hESCs summary

Cell line	Before definitive endoderm	Definitive endoderm induction	Hepatic specification	Hepatic differentiation	Hepatic maturation	Special evaluation	References
H1, H9	-	albumin fraction V (0.5mg/mL) + Activin A (100ng/mL) for 1d. Followed Activin A (100ng/mL)+ ITS for 2d	FGF4 (30ng/mL) +BMP2 (20ng/mL) for 5d	HGF (20ng/mL) for 5d	OSM (10ng/mL) +Dex (0.1uM) for up to usage	HIV-HCV pseudotype entrey and *in vivo* study by transplanted DHH into CCI4 treated SCID mice	(Cai et al. ,2007) [8]
H1, H9	-	Activin A (100ng/mL) + Wnt3a (50ng/mL) for 3 d	1%DMSO, 20% SR for 4 d	-	HGF(10ng/mL), OSM(20ng/mL) for 7d	Transplantation of Differentiated hepatocytes into NOD-SCID mice	(Hay et al., 2007) [108]
H1, H9	-	Activin A(100ng/mL), 0.5% FBS, RPMI for 3 d, 2% KOSR instead of FBS for 2 d	FGF4(10ng/mL), HGF(10ng/mL), 2%KOSR for 3d	FGF4 (10ng/mL), HGF(10ng/mL) for 3d	FGF4(10ng/mL), HGF(10ng/mL), OSM(10ng/mL), Dex(0.01uM) for 9d	Transplantation of Definitive endoderm into NOD-SCID mice	(Agarwal et al.,2008) [126]
H9	Embryoid body formation 2 days	Activin A(100ng/mL), FGF2(100ng/mL) for 3d	HGF(100ng/mL), 1%DMSO for 8d	-	Dex(0.01uM) for 3d	Transplantation of Differentiated hepatocytes into uPA-SCID, NOD-SCID	(Basma et al., 2009) [129]

Cell line	Before definitive endoderm	Definitive endoderm induction	Hepatic specification	Hepatic differentiation	Hepatic maturation	Special evaluation	References
SA001, SA002, SA002.5, SA167	-	Activin A(100ng/mL) and FGF2(4ng/mL) or Wnt3a(50ng/mL) for 5 d	BMP2(50ng/mL), BMP4(200ng/mL), FGF1(100ng/mL), FGF2(5ng/mL) for 11d	-	OSM(10ng/mL), HGF(20ng/mL), Dex(0.1uM) for 27d	-	(Brolen et al.,2009) [107]
H9	-	Activin A(100ug/mL)+ NaB(0.5uM) for 3-6d	FGF4 (20ng/mL), HGF (20ng/mL), Dex(0.1uM), BMP2/4 (10ng/mL both), 0.5% DMSO for 10-14d	-	FGF4(20ng/mL), HGF(20ng/mL), OSM(50ng/mL), Dex(0.1mM), 0.5% DMSO	Drug metabolism evaluation	(Duan et al. 2010) [128]
H9	Activin A (10ng/mL), FGF2 (12ng/mL) for 2d	Activin A(100ng/mL), FGF2(20ng/mL), BMP4(10ng/mL), LY294003(0.1uM) for 3d	FGF10 (50ng/mL) and/or retinoic acid(0.01uM), SB431542(0.1uM) for 5d	-	FGF4(30ng/mL), HGF(50ng/mL), EGF(50ng/mL) for 10d	Transplantation of Differentiated hepatocytes into uPAxRag2gammac	(Touboul et al.,2010) [127]

Cell line	Before definitive endoderm	Definitive endoderm induction	Hepatic specification	Hepatic differentiation	Hepatic maturation	Special evaluation	References
H1	-	NaB (1mM) and activin A (100ng/mL) for 2d, then Nab (0.5mM) and activin A (100ng/mL) for 3d	1% DMSO for 7 d		HGF (10ng/mL) and OSM (20ng/mL) for 7d	-	(Hay et al.,2008) [58]
H9	-	Activin A(100ng/mL)	BMP4 (20ng/mL)+FGF4 (20ng/mL)	-	HGF, OSM (20ng/mL), DEX (0.1uM)	-	(Takayama et al., 2012) [145]
H9	-	Activin A(100ng/ml), b-FGF(8ng/ml), Wnt-3a(25ng/ml) for 4d	FGF 10 (50ng/ml) for 2 d	FGF10(50ng/ml), RA(0.1uM), SB431542(1uM) for 3d	FGF-4 (30ng/ml), EGF (50ng/ml), HGF (50ng/ml) for 10d	Tested both HCVser, HCVcc	(Wu et al., 2012) [4]

Chapter 2: Differentiation of human embryonic stem cells and their *in vivo* application for Hepatitis C viral production

2.1 Introduction

Chronic Hepatitis C is a global problem caused by the Hepatitis C virus (HCV), a single-stranded RNA virus of the Flaviviridae family [146,147]. The World Health Organization estimates that approximately 170 million individuals globally are serologically positive for HCV. All three main strains of hepatitis A,B and C affect 550 million people worldwide. In 2007, it was estimated that Canada had approximately 242,500 HCV-infected individuals. Chronic HCV has extremely high morbidity and mortality rates caused by fibrosis, cirrhosis, and hepatocellular carcinoma, which are the most common indications for liver transplantation. The current treatment modalities are only partially effective and are costly, with many unwanted side effects. Interferon A and ribavirin are effective in only 50% of cases of HCV genotype 1, made evident by sustained biological responses and clinical signs of improvement [1]. Scientists are developing several drugs that have been advanced for use in clinical trials without efficacy testing on animal models. Accessible animal models are needed to mediate mechanistic studies of HCV, and for developing therapeutic and/or preventative vaccines [146,147].

HCV's natural hosts are limited to humans, but can be experimentally transmitted to chimpanzees [1,148], and experiments on these species are limited because of ethical concerns, limited accessibility, genetic variability, and high cost; therefore, several notions have been taken to generate suitable small-animal models in the last 20 years [146]. One of these approaches was to humanize mouse liver by xenoengraftment with

primary human hepatocytes (PHH). These mice models were primed to simulate a state of severe liver dysfunction and immune suppression, which would allow successful engraftment to the failing liver and proliferation without the risk of failure by rejection. Donor cells are commonly transplanted through the spleen that allow the cells to migrate into the liver using the portal venous system.

In the severe combined immune deficient /urokinase-type plasminogen activator (SCID/uPA) animal model, the transgenic mouse acquired the urokinase gene, which was influenced by the human albumin promoter/enhancer that resulted in a faster hepatocyte spread and death rate. The SCID/uPA mice were created by crossing a uPA transgenic mouse with a SCID mouse [149]. The hepatocytes harvested from human donors were then transfused into the SCID/uPA mouse. Eventually, the hepatocytes expanded in situ, replacing the mice apoptotic liver cells [2].

In 2001, Dr. Norman Kneteman (University of Alberta, Department of Surgery) developed a mouse model capable of supporting HCV infection *in vivo* by transplanting and expanding the PHH into the livers of SCID/uPA mice [2]. This model was a breakthrough in HCV research and has proven to be predictive of clinical outcomes using emerging antiviral drugs [150]. Despite the potential of this model, it remains complex, and costly. Limitations of this model are the supply of PHH, which must come from human donors; the technical difficulties associated with PHH isolation; and difficulty in proliferation *ex vivo*, which restricts expansion and genetic modification. Moreover, variability in donor liver condition (age, sex, past medical history, etc.) leads to inconsistent results. While several companies now provide regular commercial access to cryopreserved primary human hepatocytes, these are costly and have substantial variability batch to batch as well. Most of these problems can be avoided if we develop a noncancerous, renewable, and physiologically relevant

cell type that can substitute for PHH. Emerging stem-cell technologies might offer this opportunity.

Human embryonic stem cells (hESCs) are pluripotent cells isolated from the innermost cell mass of blastocysts during early embryonic development [3]. These cells are capable of self-regeneration and differentiation into any adult cell type in the human body. In the last few years, several centers around the world have successfully generated differentiated human hepatocytes (DHH) from hESCs *in vitro* [4,8,151-153]. These DHH were reported to be susceptible to HCV infection *in vitro*[8]; however, they can be genetically manipulated in the pluripotent stage to create DHH that are resistant to HCV infection [4]. Therefore, it is possible that DHH can substitute for PHH in the SCID/uPA mouse model, and this model will open the door to a new future in which scientists will be able to test antiviral medications and different host factors that play a major role in the resistance of hepatocytes to HCV, a life-saving therapy.

2.2 Materials and methods

To perform these experiments, full approval from the University of Alberta Care and Use Committee, HERO and the Stem Cell Oversight Committee were obtained.

2.2.1 PHH, hESC line (H9), growth factors, and chemicals

The following were used for the experiments: StemPro serum free medium kit (DMEM/F-12 with Glutamic, StemPro hESC Supplement, BSA 25%), catalogue #A1000701; basic FGF (b-FGF), catalog #phG0024; Geltrex, catalogue #A1413301; Accutase, catalogue #A11105-01; L-glutamine, catalogue #25030-081; and b-mercaptoethanol, catalogue #A11105-01 from Invitrogen Corporation (Carlsbad, California, USA). FGF-10, catalogue #100-26; FGF-4, catalogue #100-31; EGF, catalogue #AF-100-15; and HGF, catalogue #100-39, from PeproTech (Rocky Hill, New Jersey, USA). SB 431542, catalogue #S4317, and retinoic acid, catalogue #R2625, from Sigma-Aldrich Co. (St. Louis, Missouri, USA). Wnt-3A, catalogue #03-0009, from Stemgent Inc. (San Diego, California, USA). Activin A, catalogue #338-AC-010, from R&D Systems (Minneapolis, Minnesota, USA). Probumin, catalogue #810683, from Millipore Corporation (Billerica, Massachusetts, USA). DMEM/F12, catalogue #10-103-CV, from CellGro (Manassas, Virginia, USA). hESC line (H9) and UG supplement custom formulation provided by Dr. Hengli Tang (Florida State University, Tallahassee, Florida, USA). Cryopreserved human hepatocytes, product number #M00995-p, from Celsis, Inc. (Baltimore, Maryland, USA). A list of the primary and secondary antibodies used is provided in Table S-2.

2.2.2 Direct differentiation of hESCs into hepatocytes

A multistage, serum-free protocol was used to differentiate hESCs into human-like hepatocytes using chemically defined media as described by Wu et al. [4]. hESCs were differentiated first into definitive endoderm, then hepatoblasts, and finally into immature and mature human-like hepatocytes (DHH). Cell cultures were maintained at 37^{o}C in humidified air containing 5% CO_2 (Figure 2.1). The differentiation process and the chemically defined media used for each stage of the differentiation process are detailed in the supplementary information.

During differentiation, the cells were monitored by indirect immunofluorescence that was used to observe for various expressed markers representing each stage of the differentiation process. hESCs (d 0) and definitive endoderm (d 4) were tested for pluripotency marker OCT4 and the definitive endoderm marker chemokine receptor type 4 (CXCR4), respectively, while cells in the last stages (d 10–19) were examined for different hepatocyte markers such as albumin, alpha-fetoprotein, cytokine 18 (CK18), and cytokine 7 (CK7). A detailed protocol of these immunohistochemical procedures is provided in the supplementary information.

2.2.3 Cell count/viability

The trypan blue exclusion method was used to assess cell viability. Cell suspensions (40 uL) were mixed with 40 uL trypan blue and placed into a hemocytometer to enable a count of the cells using a microscope. Live and dead cells in the 16-square grid in a single field were counted using the 10X microscope objective. Viable cells appeared clear and dead cells appeared blue.

2.2.4 Transplantation into SCID/uPA

SCID mice homozygous for the Alb-uPA transgene (SCID/uPA) were transplanted with hESCs, DHH, or cryopreserved PHH. All mice were sheltered in a virus-free/antigen-free sitting. Under sterile conditions, SCID/uPA mice were transplanted between 5 and 10 d of age. The transplant procedure was performed under a microscope. The mice were anesthetized with isoflurane after which the surgical plane was confirmed by toe pinching and the mice were taped in the right lateral decubitus position. A small incision was made in the left subcostal area. Using curved forceps, the spleen was gently pulled from the body through the incision and a small needle connected to an infusion line was inserted at a depth of few millimeters. Assistance provided a gentle pressure to the syringe filled with 0.5–1 x 10^6 cells. After withdrawal of the needle, a surgical clip was positioned at the base of the inferior pole of the spleen. The spleen was pushed gently back into the mouse, the skin incision was closed by a simple stich using 6-0 VICRYL suture, and glue was placed over the incision.

The mice were placed in a heated recovery cage until they recovered from the anesthesia, after which they were returned to their cages. Each cage contained a single mother with her pups. The mice were put under close observation for the first 30 min after surgery to ensure that the mother accepted her babies.

2.2.5 HCV virus inoculation

Transplanted mice were inoculated with serum obtained from HCV-positive patients (HCVser) at 6 weeks post-transplant. Thawed infectious serum (2.87 x 10^5/50 uL) was injected intraperitoneally.

2.2.6 Evaluation of engraftment success by detection of hAAT in mice serum

After 6, 8, and 16 weeks, post-transplantation serum samples were collected and tested for hAAT by quantitative ELISA assay, conducted by KMT Hepatech, as previously described [150]. In brief, high binding plate wells were precoated with goat anti-hAAT antibody (1:1,000 in carbonate–bicarbonate buffer) overnight at 4^{o}C. The next day, the wells were rinsed three times with TBS solution and blocked with 5% milk buffer for 2 hr at room temperature. Serial-diluted samples of mouse serum were added to the precoated plate for 2 hr at room temperature, after which the wells were rinsed three times using TBS solution. A secondary antibody (linked to horseradish peroxidase) (1:1,000 diluted with milk buffer) was added for 2 hr at room temperature. After the plate was rinsed three times with TBS, a developing solution containing 3.3',5.5' tetramethylbenzidine (TMBD)was added, which allowed signal detection. After 7 min, sulfuric acid solution was added to stop the reaction and the plate was read using a microplate reader at 450 nm within 30 min of color developing. The data were analyzed using SoftMax Pro. Appropriate controls were used for positive control human serum and negative control non-transplanted SCID/uPA mouse serum.

2.2.7 Detection of HCV in mouse serum by qRt-PCR for viral RNA

After 4 and 10 weeks post-inoculation, mice serum was examined for evidence of HCV RNA by qRT-PCR conducted by KMT Hepatech, as previously described [2]. After isolation of viral RNA using the guanidine isothiocyanate–silicon (GTC) method, first-strand cDNA was synthesized by using a primer targeting the HCV non-coding region (60 min at 37^{o}C, 5 min at 95^{o}C, and 4^{o}C thereafter). Real time-PCR was conducted using Taqman® Gene Expression Master Mix in 96-well dishes with a total reaction of 50 uL containing 9 uL diluted samples, 2 uL backward primer, 2 uL forward primer, 2 uL probe Fam, and 10 uL ddH2O. Amplifications and

analysis of cDNA fragments were carried out using the RT-PCR 7300 system. Cycling conditions were initial denaturation at 50^{o}C for 2 min and 95^{o}C for 10 min, followed by 45 cycles consisting of 15-sec denaturation intervals at 95^{o}C and a 1-min interval for annealing and primer extension at 60^{o}C. The primers used are listed in Table 2-1.

2.2.8 Detection of human genomic DNA within transplanted mice liver by PCR

A PCR- based assay was used for detection of Alu repeat sequence in livers from transplanted mice [2]. Liver tissues were harvested 16 weeks after transplantation with DHH. Half of the liver was frozen in liquid nitrogen and stored at -80^{o}C for detection of Alu repeat sequence using PCR assay. The remaining liver tissue was fixed and embedded in paraffin for detection of Alu repeat sequenced with in situ hybridization (see below).

Genomic DNA was isolated by following the instructions in the DNeasy Blood and Tissue Kit provided by QIAGEN (Valencia, California, USA). PCR was conducted using Master Mix in the PCR tube with a total reaction of 100 uL containing 10 xPCR buffer, 10 mM dNTP, 50 mM $MgCl_2$, 10 uM forward primer, 10 uM backward primer, 200 ng template DNA, dH_2O, and Taq DNA polymerase. The Alu repeat sequence was amplified by PCR using thermal cycling conditions where initial denaturation for 5 min at 95^{o}C followed by 39 cycles for denaturation, annealing, and primer extension (30 sec at 95^{o}C, 30 sec at 50^{o}C, and 30 sec at 72^{o}C, respectively). This was followed by an additional primer extension at 72^{o}C for 5 min, after which the reaction was held and maintained at 4^{o}C, as previously described [2]. Amplified DNA was mixed with a loading buffer and added to 2% agarose gel for electrophoresis. Positive controls were mice transplanted with PHH and human liver

samples. A negative control was a non-transplanted mouse. The primers used are listed in Table 2-1.

2.2.9 Detection of human DNA within transplanted mouse liver tissue by in situ hybridization

Immunohistology was performed on paraffin-embedded section slides. Liver samples were fixed and embedded in paraffin, and 8-um sections were cut. A super-sensitive one-step polymer-HRP in situ hybridization detection system kit by BioGenex (Fremont, California, USA) was used to detect human target DNA sequences in paraffin-embedded tissue sections. An alu probe was used to detect the Alu repeat sequence of the human genome in the nucleus. (A full detailed protocol is attached in the supplementary information.) In brief, proteinase K was pretreated to expose a targeted DNA sequence. A fluorescein Alu probe was used to hybridize the exposed Alu repeat sequence. Downstream detection of the probe was done using anti-fluorescein antibody and polymer-HRP secondary antibody conjugate, followed by adding DAP to HRP to form a color reaction and a brown precipitation in the nucleus. Hematoxylin helped to stain the remaining structure of tissue. The tissues were examined under a microscope and pictures were taken. Mice transplanted with PHH and non-transplanted mice served as positive and negative controls, respectively.

2.3 Results

2.3.1 In vitro differentiation of hESCs into hepatocytes

OCT4 serves as a marker for pluripotency, whereas CXCR4 is a marker for definitive endoderm. Before differentiation (d 0), cultured hESCs were OCT4 positive and CXCR4 negative. By d 4, increased CXCR4 expression was observed, whereas very little OCT4 expression remained (Figure 2-2). These results indicate successful differentiation into definitive endoderm. Differentiation into hepatoblasts was evaluated by examining alpha-fetoprotein and CK7, whereas hepatocyte-like cells were evaluated by examining alpha-fetoprotein and albumin expression as well as CK18 expression, which is a marker for the hepatocyte phenotype. By d 10 of the differentiation process, cells had not yet begun to express albumin; however, albumin expression was noted at later time points. Alpha-fetoprotein expression was noted to increase from d 10-15 of the differentiation process, then began to decrease thereafter. A gradual decline in expression of CK7 was noted over the course of differentiation (Figure 2-3). CK18 expression was only stained for at d 15 and was positive for further hepatocyte phenotypic confirmation (Figure S1). Taken together, these results are a strong indication that the differentiation of hESCs into hepatocyte-like cells *in vitro* was achieved.

For transplantation into SCID/uPA mice, cells were harvested at different time points (d 0, 10, 13, 15, and 18) to account for the possibility that cells earlier in the differentiation process might engraft and proliferate better within the SCID/uPA background than more mature cells, and might also differentiate into hepatocytes in an *in vivo* setting. Trypan blue was used to assess DHH cell membrane integrity just before the transplantation procedure and again staining the remaining cells that were left out after transplantation was completed to ensure viability throughout

transplantation. Cell viability was >96–98% before transplantation, and this viability was maintained over the course of the transplantation period, establishing that each mouse received cells with similar (and high) viabilities.

2.3.2 Transplantations into SCID/uPA mice and infection with HCV

For the purpose of discussion, all cells enrolled into the differentiation program will be referred to as DHH regardless of the time point at which they had been harvested and used for transplantation. Using a well-established procedure in our lab, DHH was transplanted into SCID/uPA mice to assess whether these cells could substitute for PHH in this mouse model. Thirty-five SCID/uPA mice were transplanted with undifferentiated (d 0) hESCs (n = 7 mice), PHH (cryopreserved PHH, n = 9 mice), or DHH collected at different time points (n = 19 mice). These are summarized in Table 2-2. Five mice were transplanted with DHH collected on d 10, six mice with DHH collected on d 13, four mice with cells collected on d 15, and four mice with DHH collected on d 18. Eight mice were found dead or missing within the first 48 hr. These were mice transplanted with DHH from d 10 (n = 2), d 13 (n = 4), d 15 (n = 1), and d 18 (n = 1), arguing against the possibility that death was caused by any one cell type. All mice that were transplanted with undifferentiated hESCs were sacrificed after 23 d because of abdominal distention. At weaning (6 weeks post-transplantation), the remaining mice were inoculated with serum obtained from a patient with chronic HCV infection. Two mice were missing after 4–6 weeks post-inoculation with HCV. These were mice that had been transplanted with d-13 and d-15 DHHs, arguing against the possibility that any one cell type contributed to this outcome. From the 19 mice that had been transplanted with DHH, 9 mice survived to the planned experimental endpoint (16 weeks post-transplant).

2.3.3 HAAT ELISA assay

A serum-based ELISA assay was used to detect human alpha-1-antitrypsin (hAAT) in transplanted mice. This is routinely used by our lab for assessing engraftment success in SCID/uPA mice that had been transplanted with human PHH. Table 2-3 shows the results of the serum hAAT ELISA assay for the DHH, hESCs and PHH transplanted groups. The DHH group showed undetectable levels of hAAT in the serum after 6, 8, and 16 weeks post-transplantation. Interestingly, mice transplanted with hESCs showed low levels of hAAT secretion (11–24 ng/mL) in serum samples obtained at sacrifice (23 d post-transplant). SCID/uPA mice that had been transplanted with PHH secreted considerably higher levels of hAAT values ranging from 229 to 1515 ng/mL and 265 to 1405 ng/mL in both weeks 6 and 8, respectively. Typically, the levels of human engraftment in these mice are considered to be sufficient to support HCV infection. By contrast, mice transplanted with DHH did not have appreciable levels of hAAT at any of the time points tested. The engraftment of hESCs was not unexpected because it is well known that these can cause teratomas; however, these results do indicate that the transplanted hESCs are able to differentiate into a cell type that is capable of expressing and secreting hAAT. Given the aggressive proliferation of these cells in the mice, data suggest that only a small subpopulation of transplanted hESCs were able to differentiate into hepatocytes in SCID/uPA mice. These results also indicate that despite their high viability before transplantation, DHH cells either did not survive long enough to engraft to the livers of SCID/uPA mice, or were incapable of further expansion to sufficiently replace the mouse liver parenchyma with DHH capable of appreciable hAAT secretion. Another possibility is simply that DHH did engraft and replace the mouse parenchyma, but unlike PHH, DHH do not synthesize and secrete appreciable levels of hAAT.

2.3.4 Production of infectious HCV in transplanted mice serum

To investigate whether transplanted mice were able to establish HCV infections, serum samples were examined for HCV RNA using a standardized assay (performed by KMT Hepatech). Table 2-4 shows the values of HCV titers after 4 and 10 weeks in DHH groups. All mice that had been transplanted with DHH had undetectable HCV titers while several (3/9) mice that had been transplanted with PHH showed detectable HCV RNA levels at 4 weeks post-infection.

2.3.5 Human chimerism testing by PCR

Genomic DNA was obtained from the harvested livers of transplanted mice and used as template DNA for PCR detection of the Alu repeat sequence (Figure 2.4). The Alu repeat sequences are repetitive elements found only in primate genomes and are not present in mouse genomic DNA [154]. We considered the possibility that engrafted cells might preferentially localize to certain lobes of the liver, so to avoid bias from zoning, five random liver pieces (approx. 3 mm^3 each) were excised from each lobe and pooled together for gDNA isolation. Mice that had been transplanted with PHH exhibited a very strong amplicon at ~ 300 bP. By contrast, only a very weak amplicon was observed for mice that had been transplanted with DHH, which is most likely because of contamination; however, this was not tested. These results strongly indicate that the livers of mice that had been transplanted with DHH do not contain cells of human origin at 16 weeks post-transplant or contain only very low levels of such cells, and could provide an explanation for the lack of serum hAAT and HCV in these mice.

2.3.6 Human chimeric testing by in situ hybridization (ISH)

Sections from transplanted livers underwent immunohistochemical analysis to demonstrate the presence of human the Alu repeat sequence (Figure 2-5). This was accomplished using in situ hybridization with an Alu-directed oligonucleotide tagged with fluorescein isothiocyanate (FITC). Detection of oligo bound to the Alu repeat sequence was facilitated by immunohistochemical means for the detection of the FITC moiety. Mice that were transplanted with DHH on d 10, 13, 15, and 18 showed no evidence of histologically integrated human cells. By contrast, histologically integrated human cells were readily detected in the livers of mice that were transplanted with PHH or undifferentiated hESCs engraftment.

2.4 Discussion

2.4.1 Direct differentiation of hESCs into hepatocytes

Several studies reported successful differentiation of the hESCs into human-like hepatocytes using different approaches involving either using the spontaneous growth of embryoid bodies to imitate the inductive microenvironment of liver organogenesis or using a stepwise protocol that includes the growth factors and the cytokines necessary for hepatocyte development [8,58,128,155]. Here, a stepwise approach was used in a defined culture medium under feeder-free conditions. This transforming process mimics the embryological liver development. The mature hepatocytes express different liver markers, mainly alpha-fetoprotein, CK18, and albumin. The ultimate goal was to advance these cells in SCID/uPA mice livers and allow for the *in vivo* applications of the HCV virus infection.

We showed that treating the hESCs with activin A, FGF-2, and wnt3a enabled the induction of definitive endoderm, as evidenced by the expression CXCR4. In this study, we found that further differentiating definitive endoderm into hepatoblasts and immature hepatocytes by adding FGF-10, retinoic acid, and SB431542 was evident by the expression of both alpha-fetoprotein and CK7.

We also found that we could cause maturation by adding FGF-4, HGF, and EGF. In our study, various standards were used to ascertain the differentiated cells. Those standards were the ability to express albumin, alpha-fetoprotein, and CK18. These markers of evolving maturation appeared in sequence, and they were not all expressed at any one time. AFP, which is an early liver marker, was present on d 10. Whereas albumin, which is a marker of mature liver cells, first appeared on d 15. On d 18, cells expressed both AFP and albumin, with a downtrend for alpha-fetoprotein and an uptrend for albumin when compared with the previous levels noted on d 15.

However, under the present cultural conditions, the cells were not fully mature and could only be maintained up to 20 d in the differentiation process. Their immaturity was determined by their retained ability to produce alpha-fetoprotein, a property lacking in adult human hepatocytes. The results of this experiment were consistent with previous observations reported by Wu et al. (2012), in which cells expressed albumin at the end of the protocol [4].

Several previous studies reported that the hESCs could turn into human-like hepatocytes; however, none reported that the cells could be engrafted efficiently in mice models. Moreover, there were no reports that these cells could be productively infected with the HCV *in vivo*. Both processes were attempted in this experiment.

2.4.2 Transplantation of DHH into SCID/uPA mice

Here we review some reports that showed limited success or unsuccessful engraftment after the transplantation into the rodents. Cai et al. (2007) reported low efficiency engraftment into CCI/4 injured mice (non-obese, diabetic, SCID mice) livers after using a three-step differentiation protocol, spotting few cells positive for alpha-1-antitrypsin in the transplanted tissue after 2 months of intrasplenic injections[8]. On the other hand, Haridass et al. (2009) reported unsuccessful engraftment of the differentiated human-like hepatocytes into SCID/uPA mice after using the same differentiation protocol as Cai et al[156]. Duan et al. (2007) used a different approach where the differentiation of the embryoid bodies into the hepatocyte-like cells was induced using a lentivirus vector encoding alpha-1-antitrypsin. These cells were injected under a liver capsule into NOD-SCID mice (non-obese, diabetic, SCID mice). Three weeks later, the cells expressed different human liver-specific genes (albumin, hAAT, CYP1b1, and GAPDH, with the exception of AFP) [128]. Agarwal et al. (2008) transplanted definitive endoderm into NOD-SCID mice, which showed less

than 1% integration [126]; however, they were successful with the other differentiation technique of using the embryoid body and lentivirus vector. Still, direct differentiation of the hESCs into the human-like hepatocytes in the chemically defined media under feeder-free conditions and using specific growth factors and cytokines has not been reported to be successful.

Primary hepatocytes remain a scarce source due to their heterogeneity, donor limited availability, and technical difficulties faced during isolation, whereas the hESC- derived hepatocytes theoretically are readily obtainable, highly reproducible with preferred genetic malleability. In this study, DHH was transplanted into SCID/uPA mice at different time points in the differentiation protocol (d 10, 13, 15, and 18) to find the time point at which optimal engraftment occurred. To evaluate the functional engraftment of DHH transplanted cells compared with that of PHH, ELISA, and qRT-PCR tracked human hAAT secretion and the HCV production in mice serum, respectively. DHH transplanted mice failed to produce either hAAT or the HCV RNA in their serum. Furthermore, the structural engraftment was assessed to identify an Alu repeat sequence, which is limited to a primate origin, by two independent means: PCR assay and *in situ* hybridization. Neither assay showed evidence of engraftment with the DHH transplanted mice, while both consistently confirmed engraftment with primary human hepatocytes. Different engraftment tests were conducted and all failed to detect any engraftment with the differentiated hepatocytes. In addition, the HCV RNA was undetectable in the mouse serum, which can be explained by an unsuccessful engraftment of DHH into SCID/uPA mice, whereas PHHs were successfully engrafted and were able to sustain the HCV. Our results confirm the previous studies that most protocols for DHH do not yet provide the full capacity of repopulation.

Despite the promising advances *in vitro* differentiation methods, the engraftment and proliferation of the hESC-derived hepatocytes have been unsatisfactory and suboptimal in small-animal *in vivo* models. Several hypotheses are suggested and interrogated. One of them is environmentally unsuitable and the discordancy of these cells to specified extracellular matrixes, growth factors, and cytokines in the substance of the mice livers.

Second, if indeed these cells found an appropriate environment and failed engraftment, the concern rose around the phenotypic and function of these cells. Several studies have showed the ability to transform the hESCs into human-like hepatocyte cells; however, most studies conducted limited phenotypic and functional tests. It was reported that certain markers, such as albumin, are relatively easy to produce [104]. Further evaluation to compare the hESC-derived hepatic cells with PHH is necessary. In addition, as reported by Wu et al. (2012), these differentiated cells can sustain the HCV *in vitro* [4]. Despite their ability to sustain the HCV infection *in vitro* (Wu et al., 2012), DHH may be premature in comparison to the adult hepatocytes. As reported previously by Haridass et al. (2009), the fetal hepatocyte repopulation was significantly lower than that of adult hepatocytes after transplantation *in vivo* [156]. The reduced capacity of the fetal liver and DHH to repopulate after transplantation might result from differences in the engraftment efficacies. Further studies are needed.

Third, the isolated PHH might contain stromal cell types that are necessary for the repopulation and expansion in the *in vivo* environment, while DHH might lack these cells. This is supported by the observed survival of the undifferentiated hESCs in the SCID/uPA mice. In addition, Takebe et al. (2014) reported a successful transplant of functional hepatocytes after the transplantation of liver-bud. Liver- bud originated from a tri-lineage mixture of tissues: iPSCs, endothelial, and mesenchymal

stem cells [157]. Animal studies suggest that the endothelial cells can stimulate organogenesis and regeneration through the elaboration of the paracrine effect [158].

2.4.3 Transplantation of hESCs into SCID/uPA mice

A major challenge facing stem cells therapeutic approaches is their ability to proliferate unlimitedly and with an uncontrollable manner leading to the development of teratomas. This issue should be addressed meticulously before further clinical application is considered [18,159-161].

Mice transplanted with the undifferentiated hESCs that were sacrificed 23 d after transplant displayed marked abdominal distension and had gross confirmation of tumor development. The serum assays detected hAAT at much lower levels than with the PHH. The hESCs transplanted mice were confirmed to have been successfully engrafted by demonstration of the Alu repeat sequences with *in situ* hybridization. Some molecular characteristics of the hESCs, such as a rapid proliferation rate and a lack of contact inhibition, might explain why they survived in the *in vivo* model.

The manipulation of the hESCs holds a boundless potential for regenerative medicine, basic science, and clinical research. However, cell transplantation in small-animal models is a crucial step to be addressed for the progress of the stem cell-based development, which has offered inadequate data so far. In our hands, DHH were not able to substitute for the PHH in the SCID/uPA mouse model when derived from a stepwise protocol, which included critical growth factors and cytokines.

2.5 Conclusion

In conclusion, we have successfully reproduced the differentiation of the hESCs into hepatocyte-like cells using chemically defined culture media complemented with recombinant growth factors and cytokines as reported by Xu et al. (2012). Unfortunately, our *in vivo* experiments provided unsatisfactory or negative data to support that DHHs could be a substitute for PHH in the SCID/uPA mouse after intrasplenic injection. Considering recent advances in this field, it is expected that DHH with an adequate level of differentiation will be available for the biomedical and clinical applications in upcoming years.

2.6 Figures and tables:

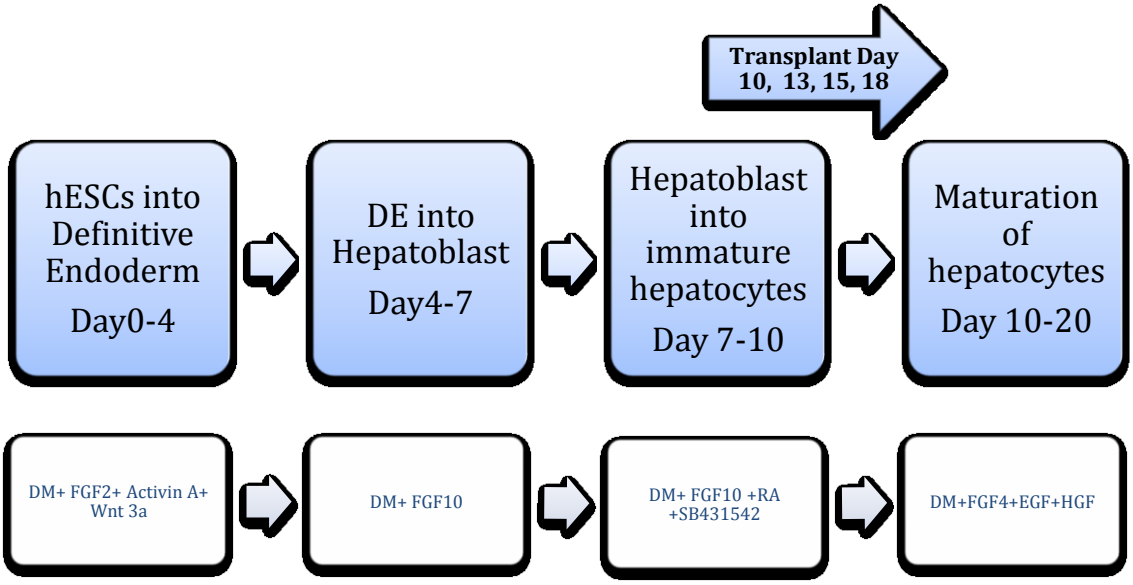

Figure 2-1 In vitro step-wise differentiation protocol

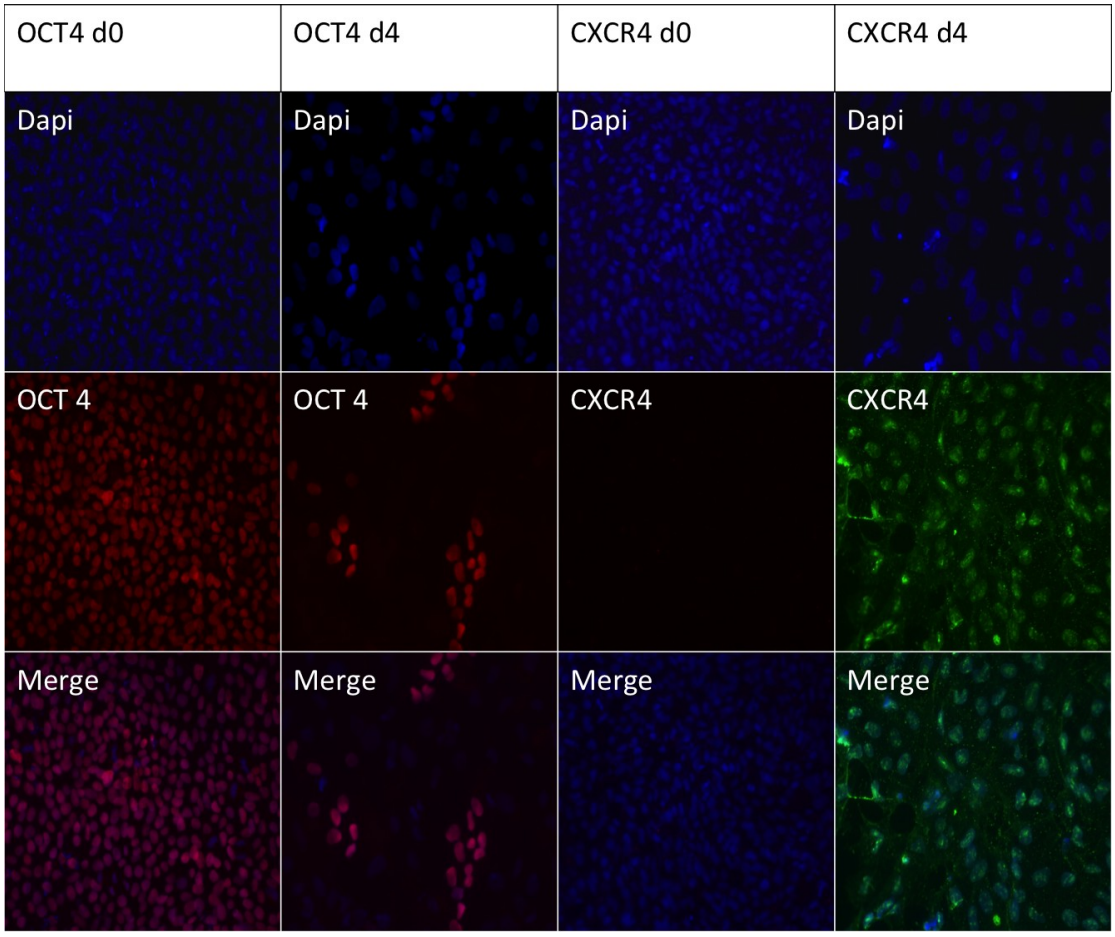

Figure 2-2 Fluorescence microscopy images for OCT4 and CXCR4 expression in undifferentiated human embryonic stem cells (Day 0) and differentiated human embryonic stem cells (Day 4)

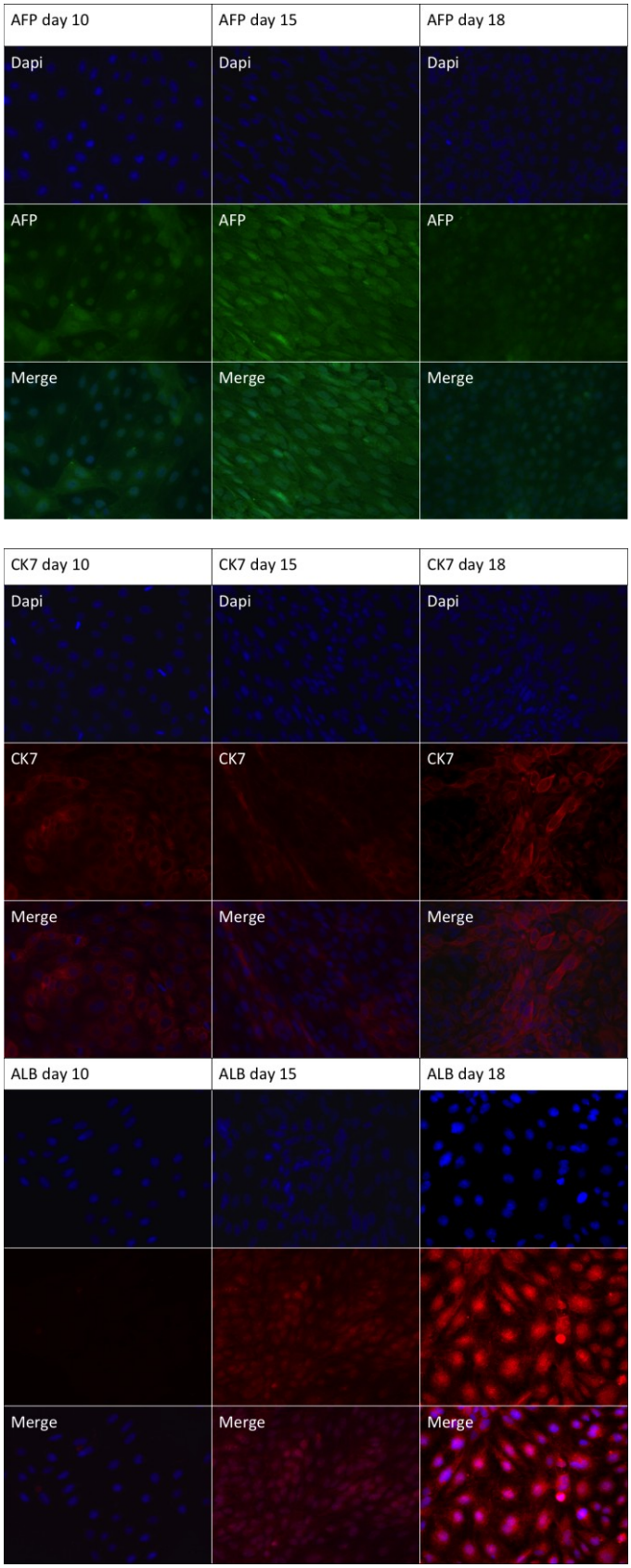

Figure 2-3 Fluorescence microscopy images for Alpha-feto protein, CK7, and albumin expression in differentiated human embryonic stem cells (Day 10-18)

Figure 2-4 PCR results for *Alu* repeats sequence

* -ve= negative control, +ve= positive control, HH= human hepatocytes

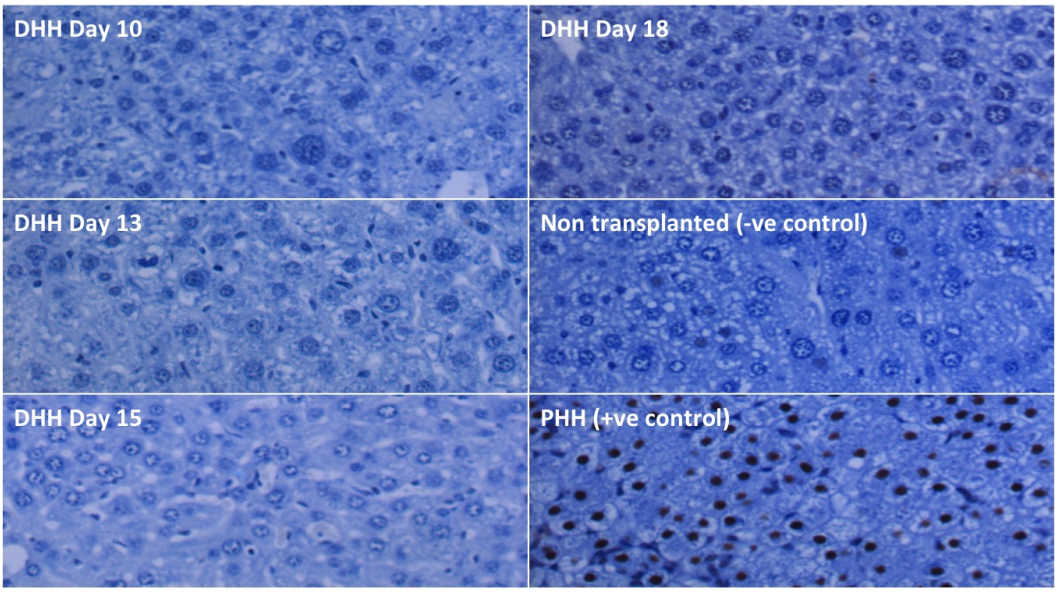

Figure 2-5 In situ hybridization results for Alu repeats sequence

*DHH= Differentiated Human hepatocytes, PHH= primary human hepatocytes

Table 2-1 List of PCR primers sequences

Gene	Forward Sequence	Reverse Sequence
Alu Sx	5′-GGCGCGGTGGCTCACG-3′	5′-TTTTTTGAGACGGAGTCTCGCTC-3′
HCV	5’-TGCGGAACCGGTGAGTACA-3’	5’-AGGTTTAGGATTCGTGCTCAT-3’

Table 2-2 Transplantation data

#	Mouse	Planned endpoint	Txp Date	Donor	# Cells/Txp
1	S767	yes	16/04/2013	DHH d10	1
2	S769	yes	16/04/2013	DHH d10	1
3	S771	yes	16/04/2013	DHH d10	1
4	S766	Missing	16/04/2013	DHH d10	1
5	S768	Missing	16/04/2013	DHH d10	1
6	S783	no	16/04/2013	DHH d13	1
7	S835	yes	29/05/2013	DHH d13	1
8	S780	Missing	16/04/2013	DHH d13	1
9	S840	Found dead	29/05/2013	DHH d13	1
10	S841	Found dead	29/05/2013	DHH d13	1
11	S784	Missing	16/04/2013	DHH d13	1
12	S749	no	26/03/2013	DHH d15	1
13	S750	yes	26/03/2013	DHH d15	1
14	S751	yes	26/03/2013	DHH d15	1
15	S748	Missing	26/03/2013	DHH d15	1
16	504	Found dead	4/4/13	DHH d18	1
17	P553	yes	17/4/2013	DHH d18	1
18	P554	yes	17/4/2013	DHH d18	1
19	P555	yes	17/4/2013	DHH d18	1
20	961	yes		H9	1
21	962	yes		H9	1
22	956	yes		H9	1
23	958	yes		H9	1
24	960	yes		H9	1
25	S851	yes	12/06/2013	Cryo	0.5
26	S852	yes	12/06/2013	Cryo	0.5
27	S855	yes	12/06/2013	Cryo	0.5
28	S862	yes	12/06/2013	Cryo	0.5
29	S868	yes	12/06/2013	Cryo	0.5
30	S869	yes	12/06/2013	Cryo	0.5
31	S871	yes	12/06/2013	Cryo	0.5
32	S872	yes	12/06/2013	Cryo	0.5
33	S873	yes	12/06/2013	Cryo	0.5

*DHH= Differentiated Human hepatocytes , H9= human embryonic stem cells, Cryo= cryopreserved human hepatocytes

Table 2-3 Human alpha-1-antitrypsin (hAAT) ELISA quantification results

Mouse	Donor	# Cells/Txp	Wean hAAT	Wean Date	8w hAAT	16w hAAT
S767	DHH d10	1	0	6 weeks	0	0
S769	DHH d10	1	0	6 weeks	0	0
S771	DHH d10	1	0	6 weeks	0	0
S783	DHH d13	1	0	6 weeks	0	0
S835	DHH d13	1	0	6 weeks	0	0
S749	DHH d15	1.5	0	6 weeks	0	0
S750	DHH d15	1.5	0	6 weeks	0	0
S751	DHH d15	1.5	0	6 weeks	0	0
504	DHH d18	1	0	6 weeks	0	0
P553	DHH d18	1	0	6 weeks	0	0
P554	DHH d18	1	0	6 weeks	0	0
P555	DHH d18	1	0	6 weeks	0	0
961	H9	1	11	23 days		
962	H9	1	24	23 days		
956	H9	1	22	23 days		
958	H9	1	12	23 days		
960	H9	1	13	23 days		
S851	Cryo	0.5	689	6 weeks	819	
S852	Cryo	0.5	343	6 weeks	1147	
S855	Cryo	0.5	1515	6 weeks	1099	
S862	Cryo	0.5	260	6 weeks	1283	
S868	Cryo	0.5	1218	6 weeks	1186	
S869	Cryo	0.5	229	6 weeks	265	
S871	Cryo	0.5	1401	6 weeks	1405	
S872	Cryo	0.5	710	6 weeks	363	
S873	Cryo	0.5	616	6 weeks	430	

*DHH= Differentiated Human hepatocytes , H9= human embryonic stem cells, Cryo= cryopreserved human hepatocytes

Table 2-4 Hepatitis C virus qRT-PCR results

Mouse	Cell	Inoculation Dose	Route	4wk HCV titer
S767	DHH 10	2.87x10^5 50ul	i.p.	0
S769	DHH 10	2.87x10^5 50ul	i.p.	0
S771	DHH 10	2.87x10^5 50ul	i.p.	0
S783	DHH 13	2.87x10^5 50ul	i.p.	0
S835	DHH 13	2.87x10^5 50ul	i.p.	0
S749	DHH 15	2.87x10^5 50ul	i.p.	0
S750	DHH 15	2.87x10^5 50ul	i.p.	0
S751	DHH 15	2.87x10^5 50ul	i.p.	0
504	DHH 18	2.87x10^5 50ul	i.p.	0
P553	DHH 18	2.87x10^5 50ul	i.p.	0
P554	DHH 18	2.87x10^5 50ul	i.p.	0
P555	DHH 18	2.87x10^5 50ul	i.p.	0
S851	Cryo	2.87x10^5 50ul	i.p.	0.00E+00
S852	Cryo	2.87x10^5 50ul	i.p.	3.55E+03
S855	Cryo	2.87x10^5 50ul	i.p.	0.00E+00
S862	Cryo	2.87x10^5 50ul	i.p.	0.00E+00
S868	Cryo	2.87x10^5 50ul	i.p.	6.04E+07
S869	Cryo	2.87x10^5 50ul	i.p.	0.00E+00
S871	Cryo	2.87x10^5 50ul	i.p.	2.90E+06
S872	Cryo	2.87x10^5 50ul	i.p.	0.00E+00
S873	Cryo	2.87x10^5 50ul	i.p.	0.00E+00

*DHH= Differentiated Human hepatocytes, Cryo= cryopreserved human hepatocytes

2.7 Supplemental information:

2.7.1 Direct differentiation of human embryonic stem cells into hepatocytes protocol:

Coating plates with Geltrex:

Invitrogen suggests using 1:100 dilutions. We have been using 1:200 dilutions per instructions of our collaborator.

For 1:200 dilution:

A one mL geltrex vial was thawed over night at 4^{o}C or kept 3 to 4 hours on ice prior to use. Thawed gletrex was mixed with one mL of DMEM/F-12 in a 15mL conical with 1:1 dilutions. Then split in to two 15mL conicals. In one of the 15 mL conicals, 14mL of DMEM/F-12 media was added, (while the other conical was frozen at -20^{o}C). This mix was then divided into five 50mL conicals. Then, 17mL DMEM/F-12 media was added to one of the 50mL conicals (the rest aliquots were stored at -20C) and mixed thoroughly. The prepared media was then used to entirely coat different dishes. The following quantity of the prepared media and corresponding dish sizes could be used: 12-well dish ~0.5mL; 6-well dish ~1mL; 60- mm dish ~2mL and a 100mm dish ~4mL. All of the previous steps were handled over ice. The coated dishes were sealed with parafilm and kept in an incubator from 1 hour up to 2 weeks.

Handling HESCs:

Human embryonic stem cells (hESCs) were provided from Dr. Hengli Tang (Florida State University, Florida, US). These cells (H9) are also commercially available from WiCell Research Institute. Cells were maintained on Geltrex coated plates which serves as a basement membrane matrix that facilitates cellular adhesion. Frozen H9 cells were thawed in 37^{o}C water bath then pipet them into 5 mL stem pro media: DMEM/F12 medium, 2% Stem pro supplement, 10% BSA, 8ng/ml b-FGF,

0.2% 2-mercapthanol, pen/strep, 1% L-Glutamine. After thawing the cells, they were centrifuged at 200G for two min and the supernatant was removed. The cells were re-suspended in stem pro media then seeded into Geltrex pre-coated plates. The cells were incubated for 5-7 days for cells to recover with a daily change of the media. The confluences of the dish were closely monitored to be between 25%-90% to prevent spontaneous differentiation.

Differentiation into Hepatocytes:

After culturing these cells for 5-7 days in stem pro-media, media was aspirated from the dish, accutase was added (1ml per 6cm dish) for a few minutes until cells were detached. Detached cells were collected into stem pro media and centrifuged at 200G for two minutes. Pelleted cells were resuspended in stem pro media and seeded on Geltrex pre-coated plates at 20-30% confluence.

Four to twelve hours after seeding, the cells were ready for hepatic differentiation. Stem pro media was replaced with basal defined medium (DM); (DMEM/F12 containing 10% Probumin, 2% UG supplement, 0.2% 2-mercapthanol, 1% L-Glutamine and penicillin/streptomycin) plus other additional growth factors on different stages. For each stage DM was used as a basic media. To initiate differentiation, media was replaced with Media A; (DM+ 100ng/ml Activin A, 8ng/ml b-FGF, 25ng/ml Wnt-3a). After 24 hours the cells were washed with D-PBS for three times and Media B (DM+ 100ng/ml Activin A, 8ng/ml b-FGF) was added with a daily change of media B for three consecutive days in total. Day 4, cells were confluent or near confluent. Cells were split (using Accutase), by a 1:4 ratio into four 6cm Geltrex coated dishes and incubated in media C (DM+50ng/ml FGF 10) for 72 hours without media change, since little growth was expected. Day 7-10, media D (DM+ 50ng/ml FGF10, o.1uM RA, 1uM SB431542) was added, changed daily and

the cells were split by a 2:3 ratio during Day 9. Day 10-18, media was changed every other day using media E (DM+30ng/ml FGF-4, 50ng/ml EGF, 50ng/ml HGF).

2.7.2 Indirect immunofluresent staining:

Cells were seeded in dishes containing coverslips, then were fixed in 4% paraformaldehyde for 10min, then rinsing cells with PBT; phosphate-buffered saline(PBS) containing 20% triton X-100 two times swiftly, then three times each for 10 minutes. After that the cells were blocked for one hour in PBTG; PBT containing bovine serum albumin and normal goat serum. Cells were incubated with monoclonal primary antibody at a dilution of (1:200) with PBTG overnight at 4^0C. Then we rinsed the cells with PBS two times swiftly, then three times each time for 10 minutes. After that, cells were incubated with a secondary antibody at a dilution (1:1000) with PBG (PBS containing bovine serum albumin and normal goat serum) in the dark for one hour at room temperature. Cells were rinsed with PBS twice swiftly, then three times each time for 10 minutes in the dark. The cells were mounted with mounting solution containing DAPI. After that images were obtained.

For all of the antibodies the protocol is the same except: 1- for the CXCR4 stain where we used PBG instead of PBTG for the primary antibody to avoid excessive background, 2- for the Oct4 stain we used PBTG instead of PBG for the secondary antibody.

2.7.3 DNA isolation:

Genomic DNA isolation kit was provided by QIAGEN (Valencia, CA) and the following is a summary according to the manufacturer's instruction.

180ul of lysis buffer (10mM TRIS-HCL,100 mM EDTA, 0.5% SDS) was mixed with the liver samples. Then 20ul of proteinase K was added and incubated overnight at 56^{o}C. After that 200ul of AL was added and mixed, followed by 200ul of 100% ethanol. The mixture was then pipetted into a DNeasy mini spin columen, fitted to a collection tube. After that the tube was centrifuged at 8000rpm for one minute. Then the collection tube was discarded or emptied. 500ul AW1 was added to a columen bed and centrifuged at 8000 rpm for one minute. After that, the collection tube was discarded or emptied. Then 500ul AW2 was added and centrifuged at 14000-rpm for 3min. Then columen was placed in a new collection tube and centrifuged for one minute at the same speed. The collection tube was discarded and was replaced with an Eppendorf tube instead. 200ul of water was added, incubated for 5min and centrifuged for one minute at 8000rpm to collect DNA/water solution. Samples were stored at 4^{o}C or -80^{o}C for long-term storage. DNA measurements were achieved using a spectrophotometer.

2.7.4 In situ hybridization:

In situ hybridization analysis was preformed using super sensitive ISH detection system kit (DF400-50K; BioGenex, San Ramon, CA). Liver tissue sections were deparaffinized, using a sequence rehydration process by dipping paraffin-embedded slides in xylene for 5min, then in fresh xylene for 5min, then in 95% ethanol for 5min, then in 70% ethanol for 5min, then in H_2O for 5 min. After that, tissue were subjected to antigen retrieval using proteinase K (100ul/slide) for 20-22 min at room temperature. Then washed with TBS-T 3 times each fore 2 min.

Tissue section slides were then incubated with 2 drops of a fluorescein-labeled Alu probe (PR-1001-01; BioGenex, San Ramon, CA), denatured at 85^{o}C for 10 minutes, and hybridized overnight at 37^{o}C in humidified chamber. After rinsing the tissue with TBST three times each for 2 min, solution A (100ul) was added twice and incubated each for 5 min, interrupted and followed by a washing step using TBST three times each for 2min. Solution B (100ul) was added twice and incubated each for 5 min, interrupted with washing step using TBST three times each for 2min. Then tissue was washed with TBST three times each for 2min. Peroxide block (100-200ul) was added and incubated for 10min, then rinsed with TBST three times each for 2min. Followed by Adding 100ul of Power Block incubated for 5min. Human Alu probe was detected using anti-Fluorescein antibody followed by HRP conjugated secondary antibody detection, each incubated for 30 minutes, interrupted by a washing step using TBST three times each for 2min. After that, a DAB substrate was added for 10 minutes to form a colored reaction. Then slides were washed six times each for 2 minutes. Hematoxylin was used as a counter staining. Slides were rinsed with a lot of water. Mountain solution was added and cover slips were applied. Then microscopic examination of the slides was conducted.

2.8 Alternative approaches:

Another approach to develop humanized small animal models was tested, using mesenchymal stem cells (MSCs).

2.8.1 MSC differentiation:

2.8.1.1 Introduction:

An interesting area in stem cell research is mesenchymal stem cells (MSCs). MSCs are multipotent stem cells. It is thought that MSCs are limited to their germ layer; however, this does not appear to be accurate. It has been proven that MSCs are able to trans-differentiate into hepatocytes. Legasse et al. showed that when these cells were transplanted into FAM mice, liver function was completely restored [9]. Petersen et al. reported that MSCs play a role in liver regeneration by confirming the presence of Y chromosome hepatocytes in the liver of female patients who received bone marrow transplants from male donors [162]. Kuo et al. reported that mesenchymal stem cell-derived hepatocytes were engrafted and repopulated up to 5% of NOD-SCID-injured mice livers [163]. In the last few years, many reports have shown successful differentiation of hMSCs into hepatocyte-like cells [164,165]. Despite the potential of the hESCs, MSCs ethically are more acceptable and the risk of developing tumors is minimal.

2.8.1.2 Methods:

The Human Mesenchymal Stem Cell Hepatogenic Differentiation Kit (Cyagen Biosciences INC., Sunnyvale, CA, product HUXMX-90101) was used to differentiate mesenchyme stem cells into hepatocytes. The manufacturer's protocol was followed. Isolation and characterization of hMSCs were provided and reported by Dr. Adetola Adesida (University of Alberta, Edmonton, AB) [166]. MSCs were obtained at passage 2 and differentiation into hepatocytes was conducted at passages 3–5.

Immunofluorescence staining for hepatocyte markers (AFP,CK18, and albumin) was conducted 13 days after the differentiation protocol. A detailed protocol for the immunofluorescence staining is in the supplementary section.

2.8.1.3 Results:

Most of the differentiated cells expressed liver markers, such as albumin, alpha feto-protein, and cell surface marker CK 18 (table 5.2). The main limitation was that most of the cells died during the differentiation process, which prevented us from transplanting these cells into SCID/uPA mice.

2.8.1.4 Conclusion:

Following the negative results of the main study (see above 2-5), we could not substitute the PHH in the SCID/uPA mice model. We tried a different approach using human mesenchymal stem cells. These differentiated hMSCs showed evidence of different liver marker expressions. Unfortunately, there were not enough cells at d 13 to conduct an *in vivo* study, and the study was stopped at this level.

2.9 Supplemental figures and tables:

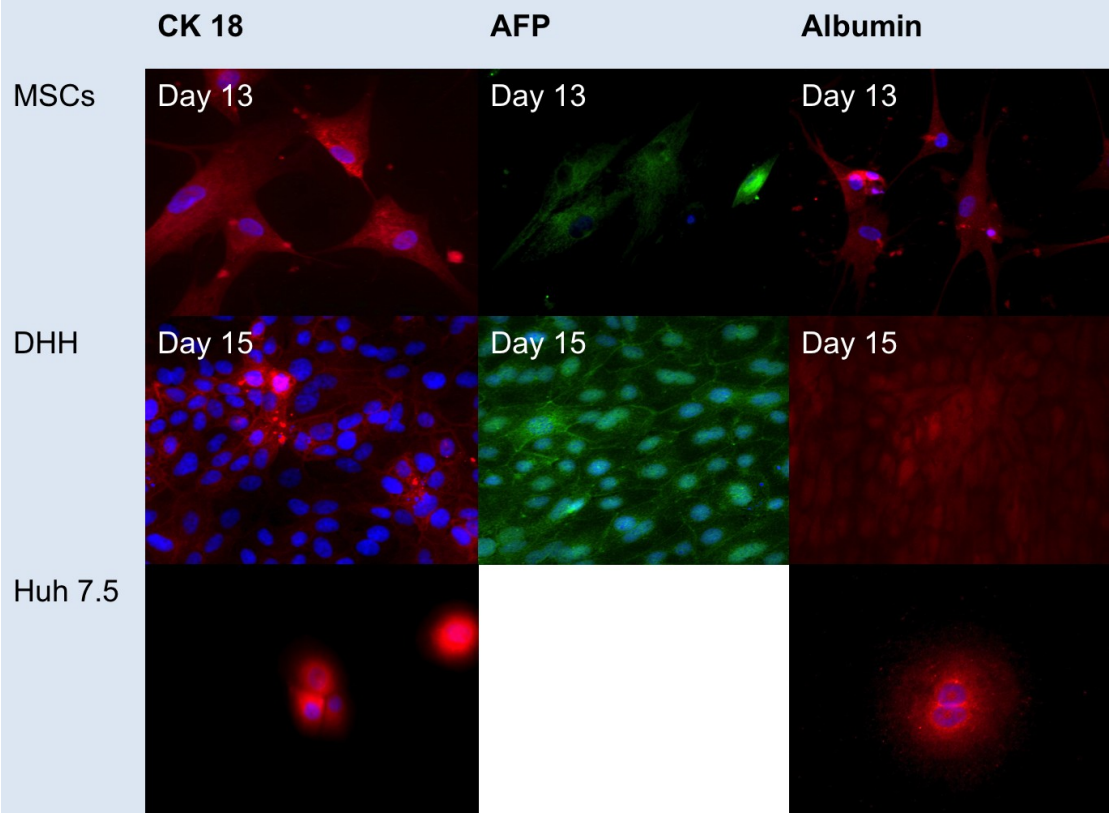

Figure S1 Indirect immunofluorescence images for CK18, AFP, and Albumin expression in differentiated human embryonic stem cells, differentiated human mesenchymal stem cells, and Human hepatoma cell line (Huh 7.5)

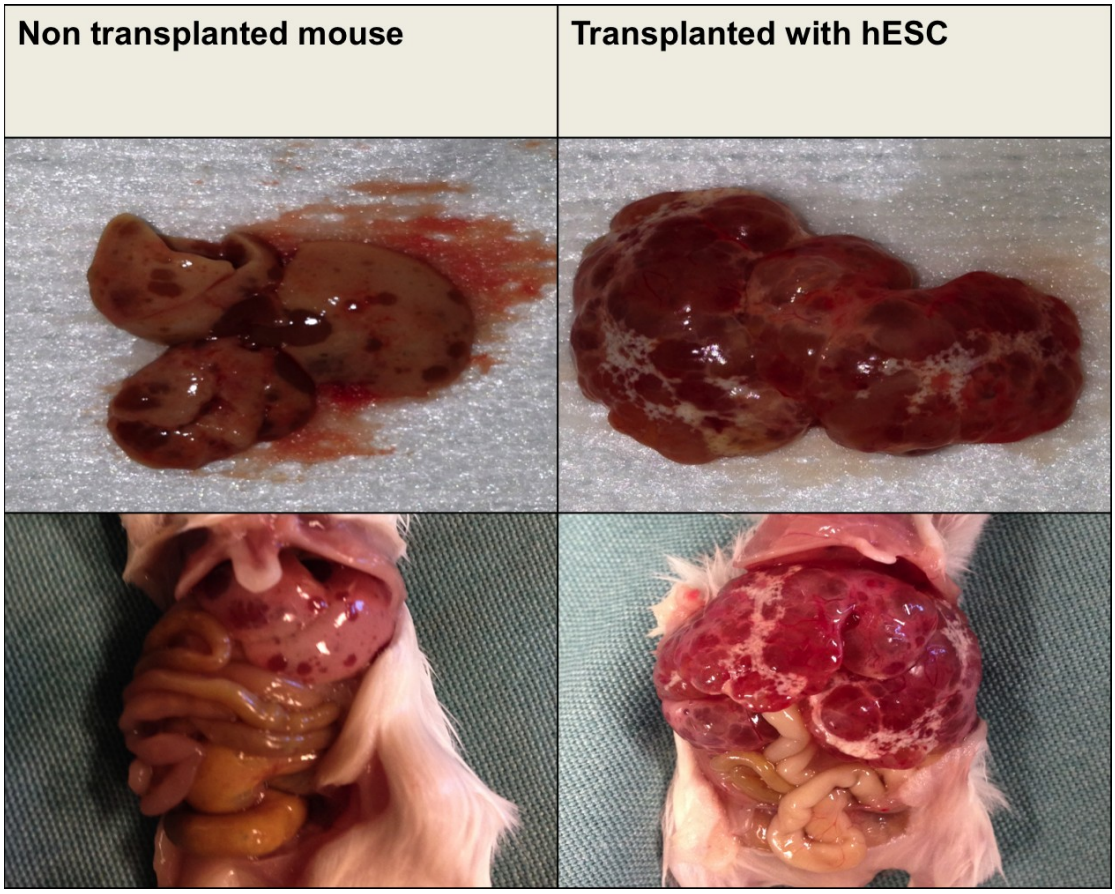

Figure S2 Images for non-transplanted mouse and hESCs in transplanted mouse

Table S1 List of primary and secondary antibodies

Primary monoclonal antibodies		
Human Oct-4	Santa Cruz Biotechnology (Dallas, Texas)	sc-5279
Human CXCR4	NIH AIDS Regents Program (Germantown, MD)	4085
Human α-fetoprotein	DAKO (Burlington, ON)	ABIN370517
Cytokeratin-7	Invitrogen (Carlsbad, CA)	18-0234
Cytokeratin-18	Provided by Lorne Tyrrell lab	
Secondary antibodies		
Goat anti mouse IgG	Life Technologies	A11029
Goat anti mouse IgG	Life Technologies	A11032
Goat anti rabbit IgG	Santa Cruz Biotechnology (Dallas, Texas)	G251

Chapter 3:

3.1 Conclusion, future Directions and challenges

During our study, we were able to differentiate the hESCs into mature hepatocyte-like cells using multi-stage differentiation protocol with certain recombinant cytokines and growth factors in a chemically defined cultural media. Unfortunately, after introducing these cells into the SCID/uPA mouse at different time points of the differentiation procedure they showed unsatisfactory or negative results to support the potential of these cells to replace the PHHs in SCID/uPA mice.

In the last decade, a great deal of studies were conducted about the differentiation of the hESCs and other types of stem cells. The advances have been great, with the largest paradigm shift being the introduction of induced pluripotent stem cells[167], which lifted the ethical concerns that were associated with the hESCs. Though the advancement towards a more efficient differentiation process is remarkable, several issues still exist and need to be resolved prior to testing these cells in the clinical trials, pharmacology and toxicology, or viral studies. For instance, the differentiation conditions should be more efficient, easy to reproduce, and comparatively affordable.

New technology may be available in the next few years that could provide the main drive of this field and be more resourceful than our limited knowledge of the differentiation process of the hepatocytes. For example, very recently Zhu et al. (2014) reported the transdifferentiation of human fibroblasts to hepatocytes and the repopulation in mouse livers[168].

In the future, many questions will need to be answered to help create a successful small-animal model and translate this information to clinical settings. Progress is headed towards the development of better protocols, which are definitely needed. During differentiation, what is the risk of tumorigenicity of the differentiated cell? At what growth stage should cells be transplanted to be effective? Should these cells be introduced in the earlier stages when they tend to proliferate rapidly and are not yet fully mature or in the later stages when the cells become more mature with limited proliferation potency? Moreover, how will we determine the different cell populations from the progenitor cells, and what is the minimal number of the grown hepatocyte cells needed to be effective after transplantation?

In relation to our study, pre- and post-transplantation studies should also be considered. Further phenotypic and functional analysis of DHH compared with PHH should be evaluated. For example, studies can look for urea synthesis, drug metabolism, and cytochrome enzyme activity. Furthermore, short-term survival analysis of DHH in a mouse liver study should be considered. Either conducting an early histology and serum analysis or using bioluminescence-imaging technology would allow us to see the fate of DHH within the first few days. This would shed more light on the cells' ability to survive and engraft.

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